EVALUATION OF THE PROTECTIVE EFFECT OF MOREL'S DISEASE VACCINE IN SHEEP

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A thesis submitted to the Graduate College,

University of Khartoum,

in partial fulfillment of the requirements

for the Ph.D. degree

Department of Microbiology

Faculty of Veterinary Medicine,

University of Khartoum,

April 2009

بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

قال الله تعالى في سورة يوسف:

و قال جل من قائلٍ في سورة الإسراء:

DEDICATION

To my mother…

Brother….

and husband …

I dedicate this work

PREFACE

This work has been carried out at the Department of Microbiology, Faculty of Veterinary Medicine, Department of Microbiology and Molecular Biology, Institute for Promotion of Animal Export Studies University of Khartoum, and the Institutes of Tropical Animals Health and Applied Biotechnology in the Tropics (IBT), University of Göttingen, Germany, under supervision and guidance of Professor Sulieman Mohamed El Sanousi.

ACKNOWLEDGEMENTS

First of all, my thanks and gratefulness are due to Almighty Allah, for helping me to finish this work successfully.

I would like to express my indebtedness and sincere thankfulness to my supervisor Professor Sulieman Mohamed El Sanousi for his keen guidance, valuable assistance, advice and encouragement.

I am also grateful to Dr. Muna El Haj (my co-supervisor) for her help. Thanks extend also to Dr. Abdulkhalig Hassan Babiker for his unlimited help during the field investigations and guidance in the laboratory work.

I would like to express my deep gratitude to Prof. A.A. Gameel for the invaluable help in post-mortem and for support and encouragement.

Special thanks are due to Dr. AbduAlazeem Yaseen and Prof. Salih Ahmed Babiker Abu Salih for their unlimited help in doing the statistical analysis.

All thanks for my small family and my husband Kamal Eldin Hassan Ali Eltom for his unlimited help in the field investigations, immunology, and molecular biology work and for language proof of the thesis.

Special thanks are due to Hashim Adallah, Hassan El Tikaid, Ali Gindeel and Gamal Tita for their patience and careful rearing of the experimental animals during the study.

My thanks are also extended to Abdel Aziz M. Elshaikh, Abbas Elssafi, Hatim Abdul-Wahab, Abdul-Kareem for technical assistance.

Thanks are also extended to technicians, the laboratory assistants and labours of the Department Microbiology, particularly Fawziyah M. Hussein, Muna Mutasim, Abdelmoneim Ramadan, Abdallah, Widad and Mawahib for their cooperation.

Nobody has been involved more closely than my family (mother, brother and husband) whom I thank for their unlimited moral support and encouragement during the period of the study.

Also I would like to express my deepest gratitude to my friends Dr. Mai Ahmed and Dr. Amani M. Khair for their encouragement.

I wish to express my gratitude to the members of Institute for Promotion of Animal Exports, especially Amal Yousuf and Kawthar H. Eltayeb.

My thanks and appreciations are also due to family of the Institute of Applied Biotechnology in the Tropics (IBT) and the Institute of Tropical Animal Health of the University of Göttingen, Germany, especially to Prof. Dr. Dr. Helge Böhnel and to P.D. Dr. Frank Gessler for their help and support during the molecular biology work. Also I am thankful to Christian Wagner and Sibyella Rheinmuth for help.

I am grateful and further indebted to my friends Afrah Mohmmed, Mawahib Abda-Allah Mahasin Showk , and Mona Abda-Allah for their moral support and encouragement.

ABSTRACT

This study was carried out to assess and evaluate the effect of Morel's disease vaccine for sheep. One hundred and seventy pus samples were obtained from sheep abscesses lymph node at meat inspection in three different abattoirs and subjected to bacteriological examination. *Staphylococcus* spp. were isolated from 68.8% of the pus samples, while *Corynebacterium* spp. were isolated from 26.5%. Mixtures of both organisms were isolated from the rest (4.7%) pus samples.

Isolated staphylococci were subjected to further identification by biochemical tests and were found to be: 63.2% *S. aureus* subsp. *anaerobius*, 21.3% *S. caseolyticus*, 11.9% *S. aureus*, and 0.9% of each of *S. simians*, *S. lugdunensis*, *S. warneri* and *S. epidermidis*.

In outbreak of the abscess disease of sheep in Alsamra village, Khartoum State, morbidity rate was 30%. Of the affected animals, 93.3% had Morel's disease, as pus cultures of which yielded *S. aureus* subsp. *anaerobius* and the rest 6.7% had caseous lymphadentitis, as pus cultures of which yielded *Corynebacterium* spp.

Comparison between the different isolates of *S. aureus* subsp. *anaerobius* using PCR based techniques (RAPD and polymorphism of *coa* and *spa* genes) showed that all Sudanese isolates were genetically identical. Complete sequence of the catalase gene of one outbreak isolate in addition to partial sequence of other two outbreak isolates, six from two different abattoirs and one reference strain was performed. Sequence results showed that all Sudanese isolates harbour a catalase gene which is distinct from the catalase gene of known reference strains suggesting that all Sudanese isolates originated from one clone. The deduce catalase-like protein encoded by the catalase gene of local Sudanese strains was found to be only 345 amino acids in length instead of 505 a.a. in *S. aureus*

subsp. *aureus* (NCTC 8325 and Newman strains) and 445 a.a in *S. aureus* subsp. *anaerobius* strain MVF213.

Ability of isolated staphylococci to induce abscess formation was tested. Except *S. aureus* and *S. aureus* subsp. *anaerobius*, none of the isolates was able to cause the clinical subcutaneous abscess of Morel's disease and only *S. caseolyticus* formed caseated lymph abscess detected on post-mortem.

Morel's disease vaccine, made according to the method of Rodwan (1996), was injected into sheep at different doses. The animals were challenged with *S. aureus* subsp. *anaerobius* using three times the minimal abscess causing dose. The minimum protective dose of the vaccine was found to be 0.5 ml boostered by 0.25 ml after two weeks. This protocol minimized the known protective dose to the half.

The protective ability of Morel's disease vaccine against abscesses due to other two staphylococci was also tested. Lambs vaccinated with Morel's disease vaccine were able withstand challenge by *S. aureus* or *S. caseolyticus* (no abscess formation in the sub cutis or in superficial lymph nodes).

Assessment of the immunity of the sheep vaccinated with Morel's disease vaccine was carried out using the Plaque Forming Cell Assay and by Opsonphagocytosis methods. Number of plaque antibody forming cells from vaccinated sheep was significantly higher ($P<0.05$) than from non vaccinated sheep. Bacteria opsonized for 2 hours by serum of vaccinated animals caused smaller subcutaneous abscesses in experimentally infected sheep when compared with that caused by non-opsonized bacteria. Also, a sharp decrease in the number of opsonized bacteria was observed indicating that the serum antibodies in response to vaccination with Morel's disease vaccine had greatly increased.

المستخلص

هدفت هذه الدراسة إلى اختبارمدى فعالية لقاح مرض الدمامل في الضأن. أخذت مائة وسبعون عينة صديد من غدد ليمفاوية من الضأن في مرحلة فحص اللحوم بثلاث مسالخ مختلفة، و أخضعت لاختبارات الفحص البكتيري. عزلت بكتيريا المكورات العنقودية من 68.8% من عينات الصديد، و البكتيريا الوتدية من 26.5%، و من متبقي عينات الصديد عزلت البكتيريا بنسبة 4.7% من كلا الجنسين.

أخضعت المكورات العنقودية المعزولة لإختبارات كيميائية للتعرف عليها و قد وجدت كالآتي: المكورات الذهبية نويع اللاهوائية 63.2%، المكورات العنقودية المجبنة 21.3%، المكورات العنقودية الذهبية 11.9%، و 0.9% من كلٍ من المكورات العنقودية القردية، و المكورات العنقودية اللقدننسية، و المكورات العنقودية الوارنرية، و المكورات العنقودية الجلدية.

أثناء حدوث وباء بالخراج في الضأن بقرية السمرة بولاية الخرطوم، بلغت نسبة المَرَاضَة 30%. و كانت نسبة 93.3% من الضأن مصابة بمرض موريل، إذ عزلت منها المكورات العنقودية الذهبية نويع اللاهوائية و البقية (6.7%) كانت مصابة بمرض السل الكاذب، إذ عزلت منها البكتيريا الوتدية.

أجريت مقارنة بين المعزولات المختلفة من المكورات العنقودية الذهبية نويع اللاهوائية بإستعمال بعض تقنيات الأحياء الجزيئية:

1. RAPD.
2. Polymorphism of *coa* and *spa* genes.

أظهرت ه ذه التقنيات أن جميع عترات المكورات العنقودية الذهبية نويع اللاهوائية المعزولة من السودان متطابقة من ناحية التسلسل الوراثي و مباينة للعترات المرجعية الأوربية.

كما أن التركيب الوراثي الكامل لمورث (جين) إنزيم الكاتاليز لواحدةٍ من العترات المعزولة من وباء مرض موريل في قرية السمرة، و كذلك التسلسل الوراثي الجزيئي له ذا المورث لإثنتين من العترات المعزولة من الوباء نفسه و لست عترات معزولات من مسلخين

مختلفين بالإضافة إلى عترة مرجعية واحدة أيد النتيجة الم ذكورة أعلاه بأن جميع العترات السودانية متطابقة وراثياً مما يشير إلى أنها تعود لعترة واحدة.

كما أجريت إختبارات عدوى تجريبية للخراف بالمكورات العنقودية المعزولة في ه ذه الدراسة و وجد أن المكورات العنقودية الذهبية نويع اللاهوائية و المكورات العنقودية ال ذهبية نويع الهوائية هما فقط القادرتان على إحداث الصورة السريرية لمرض موريل المتمثلة في الخراجات تحت الجلدية. أما المكورات العنقودية المجبنة فقد أحدثت خراجات متجبنة في العقد الليمفاوية تحت الإبطية و التي تم الكشف عنها عند فحص ما بعد الموت.

أجريت تجربة لمعرفة الجرعة الوقائية من اللقاح باستعمال اللقاح المصنع بالطريقة التي وصفها رضوان (1996) حيث حقنت الحيوانات بجرعات مختلفة من اللقاح وتم تحديها بثلاثة أضعاف الجرعة المسببة للخراج و وجد أن جرعة أولى بقدار 0.5 مل متلوة بجرعة تنشيطية بقدار 0.25 مل هي الجرعة الأقل للوقاية من هذا المرض. كما اجريت تجربة لإختبار مقدرة لقاح مرض الدمامل على الوقاية من الخراجات التي تسببها المكورات العنقودية الأخرى غير الذهبية نويع اللاهوائية. أظهرت نتائج هذه التجربة أن هذا اللقاح قد أعطى وقاية للضأن المطعمة به من الإصابة التجريبية بكلاً من المكورات العنقودية الذهبية و المكورات العنقودية المجبنة.

و لإختبار مستوى المناعة المكتسبة بواسطة اللقاح استخدم إختبار تكوين اللويحات و إختبار البلعمة. أثبت التحليل الإحصائي أن عدد الخلايا المكونة للويحات المأخوذة من حيوانات مطعمة باللقاح قد ازداد زيادة معنوية ($P<0.05$) مقارنة مع تلك المأخو ذة من الحيوانات غير المحقونة باللقاح. و عند طلاء البكتيريا لمدة ساعتين بمصل الحيوانات المطعمة باللقاح لم تستطع هذه البكتيريا سوى تكوين خراجات صغيرة مقارنة بالخراجات التي كونتها البكتيريا غير المطلية. كما ان عدد البكتيريا المطلية لمدة ساعتين قد انخفض بصورة كبيرة مقارنة مع تلك غير المطلية مما يدل على أن تركيز الأجسام المناعية المضادة المتكونة استجابة للقاح قد ازداد بصورة كبيرة في الحيوانات التي أعطيت اللقاح.

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INTRODUCTION

Morel's disease is a non-fatal contagious disease of sheep. It is caused by *Staphylococcus aureus* subspecies *anaerobius* (shortly referred to as *S. aureus anaerobius*) and is encountered in lambs between 4-10 months of age (Morel, 1911). The disease is endemic in nature with high morbidity rate and frequent relapses, but no mortality has been directly attributed to it (Bajmocy *et. al*., 1984).

In the Sudan, Morel's disease causes economical losses, especially among fattened sheep. Many shipments of exported sheep were sent back to Sudan due to this disease.

Of 936,415 sheep returned from Saudi Arabia between 1990 and 1998, 77% were rejected on the grounds of sheep abscess or Morel's disease. This has initiated a discussion about producing vaccine for Morel's disease in Sudan. At times, the Saudis may reject the whole shipment because of 1 or 2 abscess cases (Aklilu, 2002).

Twenty-eight ships containing 113,415 heads of sheep prepared for exportation to the Gulf area were rejected in March 1992. However, the rejected number of sheep increased dramatically in April 1992, (373973 heads) with a total loss of LS 7,564,600,000. The total loss of 8 years amounted to 15,142,800,000 Sudanese Dinars (Hassan, 2001).

Morel's disease was wrongly diagnosed in Saudi Arabia as "caseous lymphadenitis" or "pseudotuberculosis", which is caused by *Corynebacterium ovis*. Accordingly, a wrong vaccine was issued without any success (S. M. El Sanousi, personal communication). Hamad (1989) was the first to describe the disease in sheep in the Sudan. Rodwan (1996) produced a specific vaccine for Morel's disease using a local Sudanese strain of *S. aureus anaerobius*. El haj (2002) produced this vaccine using

the IBT bioreactor technology. In this study, we tried to evaluate the efficacy of the vaccine by using some immunological methods. Molecular biology methods were used to see possible genetic differences between local Sudanese isolates for the purpose of choosing a vaccine strain. *Staphylococcus* species other than *S. aureus anaerobius* were isolated by Noura Karamalla (1997) and Sara Bihary (2002) from some abscesses of sheep. Verifying such isolates as additional causes of Morel's disease was also aimed.

Objectives of the study

1- To survey for Morel's disease among infected animals in search of different aetiological agents for sheep abscesses.
2- To study the synergistic action of combined aetiology agents in causing the disease.
3- To investigate possible genetic differences among the local strains of *S. aureus anaerobius*.
4- To determine and evaluate the effective dose of the vaccine.
5- To improve the potency and efficacy of Morel's disease vaccine and to assess the immunity in vaccinated sheep.
6- To evaluate immunity conferred by Morel's vaccine in sheep by the use of immunological tests.

CHAPTER ONE

LITERATURE REVIEW

1.1 Abscess disease in sheep (Morel's disease)

Abscess disease is a disease of young sheep characterized by spontaneous abscess formation in subcutaneous and occasionally intermuscular tissue (Morel, 1911; Aynaud, 1927; Aynaud, 1928; Shirlaw and Ashford, 1962 and Bajmocy *et al*., 1984). According to Morel (1911) and Bajmocy *et al*. (1984), the disease is usually encountered in 8–10 months old lambs and it occurs in almost all lambs that are born in infected flocks. De La Fuente and Suarez (1985) considered it as a disease of young sheep of up to 4 months that rarely affected adults. Rodwan (1996) reported that 8 months to 3 years old sheep were generally affected, but animals around 1–5 years old were commonly affected.

The disease was also known as Morel's disease as it was firstly reported by Morel in 1911 in France (Shirlaw and Ashford, 1962 and Bajmocy *et al*., 1984). The disease was reported thereafter by other French scientists (Aynaud, 1922, 1927, 1928; Carré, 1923a, 1927; Benito and Borrel, 1957). In Hungary the disease was reported in 1983 in a flock of sheep imported from France (Bajmocy *et al.*, 1984). In Spain, Blanco-Loizelier (1985) was the first to report the disease. De la Fuente and Suarez (1985) also described an outbreak of the disease in Spain. Afnan and Hedjazi (1978) reported the disease in Iran for the first time. The disease was diagnosed in Kenya in a flock of sheep, which was built over seven years from endogenous ewes crossed with pedigree Corridale rams (Shirlaw and Ashford, 1962). In Denmark outbreak of the disease was reported for the first time by Møler *et al*. (2000) in 4–5 months old sheep

imported from France. Hamad (1989) was the first to describe the disease in sheep in the Sudan.

Goats are considered as naturally resistant to the disease. However, Valenti and Bieler (1984) reported spontaneously developing abscess disease in goats. The disease was also reported in goats in the Sudan by El Sanousi *et al.* (1989) and in Saudi Arabia by Alhendi *et al*. (1993).

Møler *et al*. (2000) reported a gradual increase in the number of affected animals that reached 40% of the flock after five months of the outbreak. The disease was mainly diagnosed in animals of good health kept for fattening (Aynaud, 1923). Also Hassan (1996) reported the relationship between the onset of Morel's disease and the fattening process as high as 62.5%.

1.2 Pathology of abscess disease

The main pathological character and the only sign of the disease is the abscess formation close to or within the superficial lymph nodes, and so, the disease is known as abscess disease (Aynaud, 1927). Aynaud (1922) and Shirlaw and Ashfford (1962) reported that the abscess developed close to - but not within - the lymph nodes, while Bajmocy *et al*. (1984) and De La Feunete and Suarez (1985) found abscesses inside the superficial lymph nodes but not around them. Occasionally, abscesses were found in the lungs (Aynaud 1922; Bajmocy *et al.*, 1984 and Hamad, 1989). Møler *et al*. (2000) found that the abscesses were closely associated with lymph nodes and the abscess wall was often fused with the lymph node capsule by a connective tissue formation that was several millimetres thick.

The most commonly affected lymph nodes are the prescapular, popliteal, inguinal and parotid lymph nodes (Morel, 1911). Aynaud (1927) and Joubert (1958) considered the angle of the jaw, the shoulder

point and the scrotum to be the predilection sites for abscesses. Shirlaw and Ashford (1962) mentioned that abscesses occurred in the following order of frequency: close to the prescapular, popliteal, parotid and anterior cervical lymph nodes. Bajmocy *et al.* (1984) noticed that suppuration occurred most frequently in the mandibular, prescapular and subiliac lymph nodes. De La Fuente and Suarez (1985) observed that abscesses most frequently located in the lymph nodes of the mandibular region (mandibular, parotid and lateral retropharyngeal) followed by superficial cervical, subiliac, popliteal, supramammary and scrotal lymph nodes, respectively. Hamad *et al.* (1992) mentioned that the distribution of suppurative lesions among naturally infected sheep involved the parotid, mandibular, prescapular, popliteal and other lymph nodes respectively. Møler *et al*. (2000) reported that the lesions predominantly occurred in prescapular region or in head: 54% of the abscesses were located in association with the superficial cervical lymph nodes followed by the parotid lymph nodes (27%) and the popliteal lymph nodes (11%).

Naturally occuring abscess is variable in size. It could be as small as a pigeon's egg or as large as an orange (Morel, 1911; Shirlaw and Ashford, 1962), two-hand fists (Aynaud, 1923), hen egg or a man's fist (Bajmocy *et al.*, 1984), or in size of a football (Alhendi, 1993) or 15 cm in diameter (Møler *et al.*, 2000). Experimentally reproduced abscess could reach up to 6.5x6.0 cm in diameter (Hassan, 1996) or 9.9x9.4 cm according to Sara Bihary (2002).

Lesions usually start small and then gradually increase in size; when ripened, the abscess ruptures expelling thin greenish yellow pus, while healing takes place after a long time (Morel, 1911; Aynaud, 1922; Bajmocy *et al.*, 1984; De la Fuente and Suarez, 1985). However, Møler *et al*. (2000) reported that the abscesses were rounded and gradually

increased in size up to 10 cm in diameter and they fistulated spontaneously expelling a viscous white- yellow odourless mass and were enclosed by a 0.2-0.5 cm thick connective tissue capsule. Aynaud (1922) mentioned that ruptured abscesses might proliferate in other points adjacent to the first site. More than two abscesses might occur simultaneously on the same animal. However, Shirlaw and Ashford (1962), and Bajmocy *et al.*, (1984) noticed that adjacent lymph nodes were usually involved a few weeks after those abscesses developing first had ruptured and healed.

1.3 Abscess formation

The sebaceous glands in the skin secrete factors that are bactericidal to Gram-positive bacteria. These factors include long- chain fatty acids and monoglycerides (Christensen, 1993; Kanai *et al.*, 1978; Shryock *et al.*, 1992 and Engler *et al.*, 1992). Fatty acids metabolizing enzyme (FAME) has the ability to inactivate some of these fatty acids by esterification to cholesterol. This may help to lower fatty acid concentrations and possibly protect the organisms from killing themselves in search for other sources of nutrients (Chamberlain, 1999). FAME appears not only to function within abscesses, but also to assist in the organism's survival in host tissues (Mortensen *et al.*, 1992 and Karbal *et al.*, 1992). Lipase enzyme produced by the organism can break down the triglycerides from sebaceous gland secretions to glycerol and fatty acids. The fatty acids obtained from breakdown of triglycerides could be utilized by the bacteria. If the fatty acid concentration gets too high, it can kill the staphylococci. The lipase enzyme is less active against the long-chain triglycerides produced in abscesses than it's against triglycerides with shorter side chains in the molecule (Muraoka *et al.*, 1982).

1.4 The causative agent of abscess disease in sheep

Joubert (1958) isolated Gram-positive micrococci from lambs affected with abscesses similar to those formerly described by Morel (1911) and Aynaud (1922, 1923). Several workers (Carré, 1927; Shirlaw and Ashford, 1962; Bajmocy *et al.*, 1984; and De la Fuente *et al.*, 1985) confirmed the relation of these micrococci to abscess disease. Benito and Borrel (1957) and Joubert (1958) proposed the name *Micrococcus pyogenes ovis* and *Micrococcus abscedens ovis* for the organism. Blanco-Loizelier (1985) and De la Fuente *et al*. (1985) demonstrated that the aetiological agent of abscess disease was catalase and benzedine negative *Staphylococcus*; they considered it to be a respiratory deficient *S. aureus*. De la Fuente *et al* (1985) reported that this respiratory deficient *S. aureus* exhibits a cell wall composition typical of *S. aureus* ATCC12600. DNA-DNA hyperdization indicated that the organism was very closely related to *S. aureus* at the species level, and because of biochemical distinctiveness (catalase and benzedine negative, negative or weak growth under aerobic conditions). De la Fuente *et al*. (1985) classified it as *S. aureus* subsp. *anaerobius*, and thereafter was included in the nineth edition of Bergey's Manual of Determinative Bacteriology (Sneath *et al*. 1986). Morel (1911), Aynaud (1922) and Carré (1923a, b) mentioned that the organism did not grow on simple media or when incubated aerobically. Shirlaw and Ashford, 1962; Bajmocy *et al.*, 1984 and De la Fuente *et al.*, 1985 mentioned that a good growth of the organism occured when cultures were incubated anaerobically under CO_2 tension, while Aynaud (1923) reported that the organism could grow aerobically when it was cultivated in Egg Yolk Agar. Shirlaw and Ashford (1962) showed that the organism did not grow aerobically even after five days of incubation, while Bajmocy (1984) noticed a pin point colonies on the

fifth day or later of incubation aerobically. Hamad (1989) and De la Fuente and Suarez (1985) noticed that after few subcultures on sheep blood agar under microaerophilic or anaerobic incubation, the organism can be adopt aerobic growth on. Møler *et al*. (2000) isolated *S. aureus* subsp. *anaerobius* in pure cultures, which were microaerophilic and appeared under aerobic condition as pin point colonies after 4–5 days. Under 10% CO_2 tension or anaerobically, 0.5–1 mm beta-haemolytic white colonies developed after 48 hours of incubation on blood agar plates.

1.5 Staphylococci isolated from sheep abscesses in the Sudan

Many authors described the organisms that cause abscesses in the Sudan. They all agreed that the organism is Gram- positive cocci, arranged in pairs, tetrads and clusters and do not grow in simple media when incubated aerobically but they disagreed in the results of biochemical reactions, especially in the haemolysin production, coagulase, pigment production and colony size.

Noura Karamalla (1997) isolated twelve different *Staphylococcus* spp. from suppurating lymph node abscesses of 85 sheep at meat inspection in Alkadaro abattoir and from subcutaneous abscesses of 15 fattened animals, viz; *S. aureus* subsp. *anaerobius* (26%), *S. hyicus* (22%), *S. caseolyticus* (20%), *S. aureus* (5%), *S. hominis* (4%), *S. dolphini* (5%), *S. sciuri* (4%), *S. cohnii* (3%) and *S. xylosus* (2%), while Sara Bihary (2002) isolated other different *Staphylococcus* spp., from lymph node abscesses of sheep at meat inspection in Omdurman abattoir, viz; *S. aureus subsp. anaerobius* (24%), *S. sacchrolyticus* (19%), *S. aureus* (10%), *S. caseolyticus* (10%), *S. hyicus* (10%), *S. simulans* (5%), *S. carnosus* (5%), *S. caprae* (5%), *S. auricularis* (5%), *S. pulvereri* (5%), *S. lugdunensis* (5%) and *S. simians* (5%).

***S. aureus*:** mainly associated with gangrenous mastitis, dermatitis and pyaemia (Timoney *et al.*, 1988). Ewes are very sensitive to experimental infection with *S. aureus* by the intramammary route; fewer than 100 bacteria are sufficient to produce clinical mastitis. In man, *S. aureus* causes a variety of suppurative (pus-forming) infections and toxaemia (Watson, 1988). It causes superficial skin lesions such as boils, styes and furunculosis; more serious infections such as pneumonia, mastitis, phlebitis, meningitis, and urinary tract infections; and deep-seated infections, such as osteomyelitis and endocarditis. *S. aureus* is a major cause of hospital acquired (nosocomial) infections of surgical wounds and infections associated with indwelling medical devices (Zierdt *et al.*, 1982; Kenneth Todar University, 2008). Also it causes food poisoning by releasing enterotoxins into food, and toxic shock syndrome by release of superantigens into the blood stream.

Staphylococcus aureus expresses many potential virulence factors such as surface proteins that promote colonization of host tissues; invasins that promote bacterial spread in tissues (leukocidin, kinases, hyaluronidase); surface factors that inhibit phagocytic engulfment (capsule, protein A); biochemical properties that enhance their survival in phagocytes (carotenoids, catalase production); immunological disguises (protein A, coagulase, clotting factor); and membrane-damaging toxins that lyse eukaryotic cell membranes (haemolysins, leukotoxin, leukocidin); exotoxins that damage host tissues or otherwise provoke symptoms of disease (SEA-G, TSST, ET); inherent and acquired resistance to antimicrobial agents. *S. aureus* can express proteases, lipase, deoxyribonuclease (DNase) and fatty acid modifying enzyme (FAME). The first three probably provide nutrients for the bacteria, and it is unlikely that they have anything but a minor role in pathogenesis.

However, the FAME enzyme may be important in abscesses, where it could modify anti-bacterial lipids and prolong bacterial survival (Kenneth Todar University, 2008).

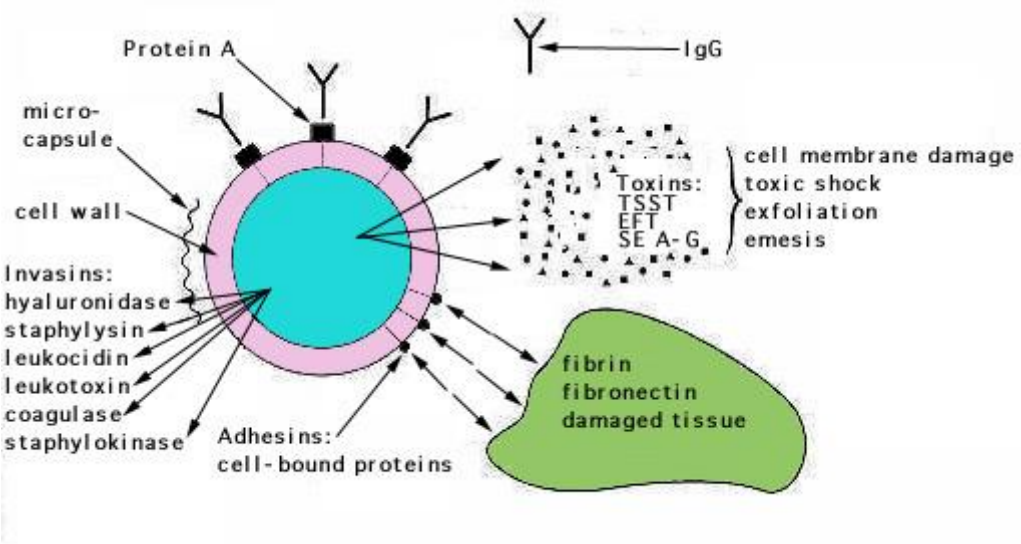


Fig. 1: Virulence determinants of *Staphylococcus aureus* (from Kenneth Todar University, 2008).

- ***Staphylococcus hyicus*:** Originally described by Sompolinsky (1950). It is a causative agent of exudative epidermitis in pigs, an infectious skin disease characterised by exfoliation of the skin, excessive sebaceous secretion, and formation of a brownish coat of exudate that may cover the entire body (Jones, 1956; Underdahl *et al.*, 1965; Devriese, 1977; Jubb *et al*., 1993; Jonsson and Wadstrom, 1993). The organism is less frequently found on the skin or in the milk of cattle (Brown *et al.*, 1967)

- ***Staphylococcus caseolyticus*:** It has been reclassified as *Micrococcus caseolyticus* (Kloos *et al.*, 1998**).** It may be found in milk and dairy products (Kloos and Schleifer, 1986).

- ***Staphylococcus hominis*:** Found living on human skin (Kloos and Musselwhite, 1975 and Kloos, 1990). Klesser *et al.* (1998) reported infective endocarditis caused by *S. hominis* after vasectomy. Although it is found on human skin, it has also been isolated as a cause of

bacteraemia in cancer patients (Bowman *et al.,* 1984).

- ***Staphylococcus delphini*:** It causes purulent skin lesions in dolphins (Varaldo *et al.,* 1988). It was also isolated from human abscess by Hind Ali (1997) and from sausage (Samia Ismail, 1997). Devriese *et al* (2005) isolated it from clinical and necropsy specimens from a cat, a dog, a horse and a parrot.

- ***Staphylococcus sciuri*:** widespread in nature, and they can be isolated from a variety of farm animals, pets, and wild animals, as well as from various food products of animal origin **(**Kloos *et al.,*1997; Garcia *et al.,* 2002 and Hauschild *et al.,* 2003).

- ***Staphylococcus cohnii***: Isolated as normal skin flora (Shleifer and Kloos, 1975) and in human skin it produces small and transient population (Kloos and Musselwhite, 1975).

- ***Staphylococcus xylosus*:** Schleifer and Kloos (1975) isolated it from human skin. Carrillo *et al.* (2000) described ***S.*** *xylosus* as a coagulase negative staphylococcal species that is emerging as a new nosocomial pathogen. Also ***S.*** *xylosus* was isolated from animal products such as milk, cheese, sausage and so forth. Some times it causes nasal dermatitis in gerbils (Solomon *et al.,* 1990) and acute pyelonephritis in humans (Tselenis-Kotsowilis *et al.,* 1982). It has also been shown to be associated with epizootic fatal dermatitis in athymic nude mice (Bradfield *et al.,* 1993)

- ***Staphylococcus saccharolyticus*:** An anaerobic species, previously called *Peptococcus saccharolyticus*. Its transfer to the genus *Staphylococcus* was based on oligonucleotide analysis of 16S rRNA (Kilpper-Blaz and Schleifer 1981). It is found on human mucous membranes. Westblom *et al.* (1990) reported a single case of endocarditis caused by this organism.

- ***Staphylococcus simulans*:** Found on the skin and in urethras of healthy women. *S. simulans* has been isolated as a cause of septicemia, osteomyelitis and septic arthritis following open reduction of fractured fibula (Males *et al.*, 1985), as a cause of native valve endocarditis (Jansen *et al.*, 1992; McCarthy *et al*., 1991).

- ***Staphylococcus auricularis*:** This species is found in the human external auditory canal, but it is rarely implicated in infections (Kloos and wolfsohl, 1983).

- ***Staphylococcus carnosus***: was isolated from dry sausages (Kloos and Schleifer, 1986).

- ***Staphylococcus caprae***: was originally isolated from goat milk (Poutrel, 1984 and Nuha Elsayed, 2001). It has been recently found on human skin and in human clinical specimens (Kanda *et al.*, 1991).

- ***Staphylococcus pulvereri***: was isolated from hip infection in human (Zakrzewska-Czerwinska *et al.*, 1995).

- ***Staphylococcus lugdunensis***: Occurs commonly on human skin (Herchline and Ayers, 1991). Obeidat (1997) isolated the organism from human nose. It causes endocarditis (Walsh and Mounsey, 1990). It has also been associated with native and prosthetic valve endocarditis, skin and soft tissue cellulites, peritonitis, infected hip prostheses, osteomyelitis, vascular line infections and breast abscesses (Freney *et al.*, 1988; Etienne *et al.*, 1989; Barker *et al.*, 1991; Cormican *et al.*, 1992; Vandenesch *et al.*, 1993 and Waghorn, 1994).

Staphylococcus simians: was isolated from beef burger (Samia Ismail, 1997) and from mastitic milk from goat and sheep (Nuha Elsayed, 2001).

1.6 Identification and characterization of *S. aureus* by molecular methods

The use of nucleic acid targets with their high sensitivity and specificity may provide an alternative means of accurately identifying *Staphylococcus* species (Drancourt and Raoult, 2002).

Recently, several investigators have described DNA-based techniques for typing strains (Cuny *et al*., 1996; Gurtler and Barrie, 1995). Several molecular taxonomic methods, including DNA–DNA hybridization and 16S rRNA sequencing, as well as various PCR-based techniques, have been reported for the identification and phylogenetic study of staphylococci (De Buyser *et al.*, 1992 and Freney *et al.*, 1999). The molecular targets have been exploited for the molecular identification of *Staphylococcus* species including: the 16S rRNA gene (Bialkowska-Hobrzanska *et al.*, 1990 and De Buyser *et al.*, 1992), the tRNA gene intergenic spacer (Maes *et al.*, 1997), the heat shock protein 60b (HSP60) gene (Goh *et al*., 1996) and the *femA* gene (Vannuffel *et al*., 1999), and many other molecualar targets.

1.6.1 PCR amplification of the thermonuclease (*nuc*) gene

Staphylococcus aureus strains produce an extracellular thermostable nuclease (thermonuclease or *nuc* gene) with a frequency similar to that at which they produce coagulase (Madison *et al.*, 1983). The TNase protein has been well characterized (Thiele, 1990), and its gene (*nuc* gene), has been cloned and sequenced (Kovacevic *et al.*, 1985). The TNase is a protein with a molecular mass of 17,000 Da (Tucker *et al*., 1978). It is an endonuclease, degrading both DNA and RNA, and its enzymatic activity can resist up to 100 °C for at least 1 hour (Lachica *et al*., 1972). An enzymatic test for TNase production was used in many laboratories for the identification of *S. aureus* isolates (Lachica *et al.*,

1971). Brakstad and Maeland (1989) developed a monoclonal antibody-based sandwich enzyme-linked immunosorbent assay for detection of the *S. aureus* TNase and obtained results which indicated its specificity for *S. aureus*. These results accord with the supposition that the *S. aureus* TNase has species-specific sequence. This supposition was supported by the findings of Liebl *et al*. (1987) who used a 518-bp fragment of the cloned *S. aureus* TNase gene which specifically recognized *S. aureus* strains in a membrane-based DNA hybridization test.

Brakstad *et al.* (1992) developed a PCR test based on amplification of part the *nuc* gene for the identification of *S. aureus.* The *nuc* primer set could recognize all staphylococci identified as *S. aureus* by conventional methods, but not the other bacteria. This *nuc*-PCR could detect viable *S. aureus* cells or correspondingly low levels (0.69 pg) of extracted DNA in saline. The sensitivity of this test accords with that described for PCR with other bacteria, being between 1 and 20 CFU (Van Ketel *et al*., 1990 and Lebech *et al*., 1991) and thus it has potential for the rapid diagnosis of *S. aureus* infections by direct testing of clinical specimens, including specimens from patients with on going antimicrobial therapy.

1.6.2 Genetic characterization of *Staphylococcus aureus*

1.6.2.1 *Staphylococcus* catalase (*kat*) gene

The main phenotypic differences between *S. aureus* and *S. aureus* subsp. *anaerobius* are the weak or negative aerobic growth and the lack of catalase activity in the latter (De la Fuente *et al.*, 1985). In *S. aureus*, a correlation between catalase activity and virulence has been observed (Mandell, 1975; Kanafani and Martin, 1985), suggesting the role of catalase as a defensive mechanism against the oxygen radicals produced by macrophages. *S. aureus* subsp. *anaerobius*, however, shares with *S. aureus* the ability to produce extracellular toxins and enzymes (De la

Fuente and Suarez, 1985; De la Fuente *et al.*, 1985), which traditionally have been related to staphylococcal pathogenicity.

Catalase is a haem- containing enzyme involved in dismutation of hydrogen peroxide generated during cellular metabolism to water and molecular oxygen. Most of the catalases characterized can be classified into one of two types based on their enzymological properties: monofunctional or typical catalases, and bifunctional catalase-peroxidases (Loewen, 1992). In many bacteria, both types of catalase are present and each enzyme is encoded by a different gene (e.g. in *Escherichia coli*, *katE* and *katG* code for monofunctional and bifunctional catalases, respectively). Monofunctional catalases have been described as proteins with molecular masses of approximately 220–350 kDa and are normally formed by four identical subunits, each containing one (proto-) haem group (Haas and Brehm, 1993). Their active centre and NADPH-binding site were described in detail by Fita and Rossmann (1985a, b). Comparison of the deduced amino acid sequences of these enzymes indicates that typical catalases share regions that are highly conserved among microbial, plant and mammalian enzymes (Switala *et al.*, 1990; Von Ossowski *et al.*, 1993).

In *S. aureus*, a typical catalase with high levels of enzymatic activity and formed of four identical subunits of approximately 60 kDa has been described (Rupprecht and Schleifer, 1979; Ruiz Santa- Quiteria *et al.*, 1992).

Sanz *et al.* (2000) conducted comparative studies between the catalase genes of *S. aureus* subsp. *aureus* and *S. aureus* subsp. *anaerobius* (*katA* and *katB*, respectively). They found that *katA* consists of 1518 base pairs open reading frame coding for a protein of 505 amino acids, while *katB* consists of 1583 nucleotides long and encodes for 455

a.a. protein. These results showed that *katA* had undergone mutations, which led to generation of *katB*. These mutations were a deletion of one base pair located at 1338 bp from the initation codon, in addition to 8 silent and 6 mis-sense mutations. The deletion resulted in shift of the reading frame and premature termination of translation with subsequent generation of *katB*. Four of the 6 mis-sense mutations present in *katB* lead to non-conservative amino acid replacements, the most significant being that located at residue 317 (Prolin in *katA*→Serin in *katB*) because the affected amino acid is involved in determining the proximal haem-binding site. Lack of the catalase activity of *S. aureus* subsp. *anaerobius* is mainly attributed to these mutations (Sanz *et al*., 2000). Similarly, loss of the catalase enzyme activity in a methicillin resistant *S. aureus* strain was also attributed to mutations of the catalase. These mutations were deletion of five successive base pairs, which led to shift in the reading frame and premature termination of translation (Grüner *et al*., 2007).

1.6.2.2 DNA Polymorphism

The sequencing of the *S. aureus* genome indicated the presence of several variable number of tandem repeats (VNTR) loci, including *coa*, and *spa* (Sabat *et al.*, 2003), which have been used for analysis of polymorphism and genetic relationship in epidemiological studies.

1.6.2.3 Staphylocoagulase gene (*coa*)

Coagulase is an extracellular protein that binds prothrombin to form a complex with thrombin-like activity which coverts fibrinogen to fibrin (McDevitt *et al.*, 1992). Coagulase is produced by all strains of *S. aureus* (Kloos and Schleifer, 1986.) and it is a major phenotypic species determinant in *S. aureus*. Its production is the principal criterion used in the clinical microbiology laboratory for the identification of *S. aureus* in human infections (Goh *et al.*, 1992), and it is thought to be an important

virulence factor (Hookey *et al.*, 1998). Within the encoding gene of *coa*, repeats of 81 nucleotides can be observed, which are clearly polymorphic in both number and sequence (van Belkum *et al.*, 1998). The sizes and DNA restriction endonuclease site polymorphisms at the 3´ coding region of the coagulase gene have been utilized in PCR-based restriction fragment length polymorphism (RFLP) analysis of *S. aureus* (Hookey *et al.*, 1998) and so, typing by PCR-RFLP of this gene can be used to monitor relatedness among *S. aureus* strains (Grzegorczyk *et al.*, 2006). Particular PCR products of *coa* gene were found in some studies of approximate lengths of 600, 700, 750 and 800 base pairs (Grzegorczyk *et al.*, 2006).

1.6.2.4 *Staphylococcus* protein A gene (*spa*)

Staphylococcal protein A is a bacterial cell wall product that binds immunoglobulin G (IgG) and impairs opsonisation by serum complement and phagocytosis by polymorphonuclear leukocytes (Colburn *et al.*, 1980 and Musher *et al*., 1981). The decrease of protein A on the cell surface of *S. aureus* results in a greater number of free receptor sites for complement C3b and an increase in phagocytosis (Gemmell and O'Dowd, 1983). The *spa* gene is composed of approximately 2,150 bp and harbors a number of functionally distinct regions: an Fc-binding region, the so-called X region, and, at the C terminus, a sequence required for cell wall attachment (Frénay *et al*., 1994). The repetitive region X of the *spa* gene includes a variable number of 24-bp repeats. The number and sequence of individual repeats may differ among strains. Frénay *et al*. (1994) reported that the number of repeats has been related to the dissemination potential of *S. aureus*: strains with more than seven repeats in the X region tended to be epidemic, while the presence of seven or less repeats was indicative of a non-epidemic methicillin-resistant *S. aureus* (MRSA) strain. *Spa*

typing has been shown to be an effective and rapid method for typing MRSA. Thus, *spa* typing is used for outbreak investigation, and it may prove useful as a practical method for describing a natural population of *S. aureus* organisms (Shopsin *et al*., 1999 and Moodley *et al*., 2006).

1.6.3 Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) is based on the whole gene by restriction endoneuclease digestion. It was recognized as being one of the most discriminatory method for gene typing strains of *S. aureus*, and it has been used to investigate nosocomial outbreaks. PFGE was shown to be a useful method for investigating the source, transmission and spread of nosocomial infections and for epidemiological typing and determination of the genetic relatedness of methicillin resistant *S. aureus* strains (De Lencastre *et al*., 1996; Lemaitre *et al*., 1998 and Shimuizu *et al*., 1999).

1.6.4 Randomly amplified polymorphic DNA (RAPD)

Randomly amplified polymorphic DNA (RAPD) assays use short primers with an arbitrary sequence to amplify genomic DNA in a low-stringency PCR. These primers randomly hybridize with chromosomal sequences that vary among different strains that produce different amplification products. These products can be separated by gel electrophoresis to produce fingerprints or patterns characteristic of different epidemiological types. The method is attractive because it is simple to perform and, theoretically, can be applied to any organism (Power, 1996).

The RAPD assay, also called arbitrarily primed PCR, is rapid and technically simple (van Belkum *et al.*, 1993 and Tambic *et al.*, 1997).
It is an effective method for the epidemiological investigation of the outbreaks, and performance of typing by this method is simpler and less

time-consuming than that of typing by Pulsed-field gel electrophoresis (Tambic *et al.*, 1997).

1.7 Molecular characterization of *S. aureus* subsp. *anaerobius* isolates from the Sudan

A recent study in Sudan to compare between local isolates of *S. aureus* subsp. *anaerobius* has been carried out by El Haj (2002). She mentioned that all local isolates were genetically identical; they have the same DNA restriction pattern and they almost simulate each other in their fatty acids composition, but with regard to protein profiles she reported little differences in the number of bands which ranged from 19 to 24, with molecular masses ranging from 53.35 to 113.84 kDa.

1.8 Pathogenicity of *Staphylococcus aureus* subsp. *anaerobius*

1.8.1 Pathogenicity to laboratory animals

Laboratory animals were found resistant to experimental infection by different researchers. Aynaud (1928) found that guinea pigs injected intramuscularly or subcutaneously were refractory to experimental infection. Also he found mice to be resistant. Joubert (1958) and Shirlaw and Ashford (1962) found mice and guinea pigs were refractory to experimental infection irrespective of the route or dose administered. De la Fuente and Suarez (1985) inoculated four groups of mice intraperitoneally or subcutaneously with increasing doses, and by intramuscular and intradermal routes. They found that mice are resistant to the disease regardless of the dose or inoculation route. Aynaud (1928) and Joubert (1958) found rabbits resistant to experimental infection through all routes of administration. However, Shirlaw and Ashford (1962) obtained cellulitis at the inoculation sites in two out of four rabbits from which the organism was recovered in pure culture. Hamad (1989) also found the same findings.

1.8.2 Pathogenicity to sheep and goats

Staphylococcus aureus subsp .*anaerobius* is pathogenic for sheep, causing the abscess disease, and experimentally for goats (Aynaud, 1923, 1927, and 1928). Goats seem to have strong natural resistance to abscess disease, although they are sensitive to experimental infection (Aynaud, 1923, 1927, 1928; El Sanousi *et al.*, 1989). Aynaud (1927) found that the organism was pathogenic for sheep and goats when administered by the intramuscular, subcutaneous, intraperitoneal and intratesticular routes. Abscesses developed at the inoculation sites, but following intraperitoneal injection of the organism, abscesses developed in the abdominal muscles. The organism is not pathogenic when given by the oral or the intratracheal routes (Aynaud, 1927).

Shirlaw and Ashford (1962) obtained the disease in sheep when the organism was administered by the intradermal or subcutaneous routes. Abscesses developed after two days on animals infected intradermally and after 12 days on those infected subcutaneously. Abscesses were observed in adjacent lymph nodes at post-mortem six weeks following infection. They noticed that inoculation of the organism into animals by scarification did not reproduce the disease in sheep. However, Bajmocy *et al.*, (1984) were able to reproduce the disease by scarification, as well as by intramuscular and intravenous inoculation. Hamad (1989) observed that sheep inoculated with a culture suspension of the organism developed a local abscess, which ruptured on the ninth day of inoculation. On autopsy seven weeks following infection, he noticed thickening and cording of lymphatic vessels from the skin lesion to the adjacent prescapular lymph node. Hassan (1996) found that most of the abscesses opened by day eight after subcutaneous inoculation of sheep with viable *S. aureus* subsp. *anaerobius*. Also, he found scarification to cause

multiple microabscesses in liver with involvement of mediastinal lymph node, but no evidence of abscess formation in the superficial lymph nodes. He noticed that the internal organs of fattened sheep inoculated intravenously with viable *S. aureus* subsp. *anaerobius*, showed no apparent lesions.

1.9 Vaccination against staphylococcal infections

There is an increasing need for a safe and effective vaccine to prevent staphylococcal infections in the field. In Veterinary practices, live staphylococcal vaccines appear to be more effective than killed vaccines (Watson and Lee, 1978; Watson, 1987).

1.9.1 Live *Staphylococcus aureus* vaccines

It has been known for many years that live *S. aureus* vaccines, given subcutaneously, provide a considerable degree of protection from staphylococcal mastitis (Bridre, 1907 and Derbyshire, 1961). Vaccination with live *S. aureus* vaccine induces a small abscess at the injection site, which is grossly and microscopically quite different from the granuloma resulting from injection of killed staphylococci (Watson and Kennedy, 1981). Ruminal neutrophils appear to be membrane receptors for the Fc portion of the IgG2 molecules (Watson, 1976). Immunoglobulin G2 is the only immunoglobulin isotype, which binds cytophilically to ruminant neutrophils (Watson, 1975). Following immunization with live *S. aureus* vaccine, ruminants mount a strong IgG2 anti-*S. aureus* antibody response (Watson, 1987). Recent studies showed that systemic vaccination of sheep with a live *S. aureus* vaccine induced an immune response which was characterized by polymorphnuclear neutrophils possessing enhanced phagocytic capacity *in vitro* assay when compared with neutrophils from ewes given killed *S. aureus* vaccine intramuscularly (Watson, 1975, 1976). Furthermore, results of challenge experiments suggest that live *S.*

aureus vaccines provided stronger protection of the mammary gland than killed vaccines when both were administered systemically to sheep (Watson and Lee, 1978). However, the problem of reversion to pathogenicity, which is common among *Staphylococcus* species, led to lesser use of live vaccines (Ahmed *et al.*, 1990).

1.9.2 Killed *Staphylococcus aureus* vaccines

Killed *S. aureus* vaccines have generally stimulated long IgG1 but poor IgG2 responses and afforded relatively less protection in experimental staphylococcal mastitis models (McDowell and Watson, 1974 and Watson, 1989;). Many attempts at vaccination with bacterins and toxoids have been largely ineffective (Anderson, 1978).

Immunization by the intramammary infusion of antigen during pregnancy was partially successful in conferring protection from homologous challenge (McDowell and Watson, 1974). Systemic administration of killed vaccine proved unsuccessful (Derbyshire, 1962).

1.9.3 Cellular components as vaccines

Other potentially protective immunogenic procedures against *S. aureus* infection include the utilization of capsular components, particularly polysaccharides (CPS) as vaccines. A few strains of *S. aureus* produce a true capsule (Wilkinson, 1983). However, when growing *in vivo*, *S. aureus* produces an extracellular glycocalyx comprised largely of hydrated polysaccharides (Mayberry-Carson *et al.*, 1984; Speers and Nade, 1985). Expression of glycocalyx usually ceases when *S. aureus* is grown in conventional laboratory media and could be lost on subculture (Opdebeeck *et al.*, 1987; Sutra *et al.*, 1990).

Surface polysaccharide capsules formed by pathogenic bacteria are important virulence factors, and immunity directed towards capsular antigens is often protective (Foster, 1991). Combination of

polysaccharide with a protein carrier has been shown to enhance immunogenicity and to stimulate a T-cell response (Fattom *et al.*, 1990). It has been difficult to study capsules in *S. aureus* because of poor capsule expression when bacteria are grown in conventional laboratory media, and because of the tendency of the phenotype to be lost on subculture (Rather *et al.*, 1986). This phenomenon was shown in mastitis isolates, the majority of which formed diffuse colonies in serum soft agar (Yoshida and Ekstedt, 1968). Only 50% of *S. aureus* strains retained diffuse morphology after growth in brain heart infusion medium but more than 80% developed diffuse morphology after growth in a high carbohydrate medium (Opdebeeck and Norcross, 1983; Yoshida and Ekstedt, 1968). Rajab (1997) reported expression of the *S. aureus* subsp. *anaerobius* capsule in serum charcoal medium developed for that purpose. Some strains of *S. aureus* permanently form mucoid colonies. They form a thick capsule called a macrocapsule, which can be visualized by light microscopy with Indian ink staining (Wilkinson, 1983 and Lee *et al.*, 1987). Mucoid strains are rarely isolated from human or ruminant infections while the most natural isolates form a microcapsule which is too thin to be detected by light microscopy (Wilkinson, 1983). The macrocapsule associated with mucoid colony morphology is known to increase the virulence of *S. aureus* for laboratory animals (Yoshida and Eskstedt, 1968; Koenig and Melly, 1965; Wiley and Maverakis, 1974). The enhanced virulence of the mucoid strains is almost certainly due to impaired phagocytosis (Wiley and Maverais, 1974; Lee *et al.*, 1988; Wilkinson *et al.*, 1979). There is also strong evidence indicating that anti-capsular polysaccharide (CPS) antibodies promote phagocytosis and killing of bacteria with microcapsule (Fattom *et al.*, 1990).

Adhesion to host cells or to tissue components is an important first

step in infection by many pathogenic bacteria. Attachment of bacteria to mammary gland epithelial cells appears to be promoted by two surface associated proteins (Lindahl *et al.*, 1990). One binds to the glycocalyx fibronectin (Forman *et al.*, 1987) which is present on the surface of these cells while the other is haemoagglutinin, which promotes binding to epithelial cells and to milk fat globules (Lindahl *et al.*, 1990). Several pathogenic Gram-positive bacteria express cell-bound proteins, which are exposed, on the cell surface. These proteins include protein A (Uhlen *et al.*, 1984) and the fibrnectin-binding proteins of *S. aureus* (Signäs *et al.*, 1989) and M proteins. These proteins have several features in common including an extended structure, a hydrophobic carboxy terminus, a proline rich region and surface-exposed repeated domains which interact with host proteins (Foster, 1991).

Protein A is a major component of the cell wall of *S. aureus* (Forsgren *et al.*, 1983). It has five tenderly repeated domains, which bind to the Fc region of mammalian IgG (Moks *et al.*, 1986). This interaction inhibits phagocytosis in the presence of normal serum (Peterson *et al.*, 1977) presumably by blocking attachment of opsnins to the macrophage. Immunization with protein A might be expected to reverse the inhibition of opsonophagocytosis. In this regard, some protection against bovine mastitis was obtained with a protein A vaccine (Nickerson *et al.*, 1985), whereas passive immunization of infant rats with rabbit anti-protein-A serum did not protect against staphylococcal infection (Greenberg *et al.*, 1989).

Protection against lethal gangrenous mastitis in rabbits was conferred by immunization with a toxoid derivative, but no protection was obtained against the chronic form of the disease (Adlam *et al.*, 1977). Derbyshire (1960) recorded only a mild transient reaction in the form of

neutrophil in the mammary glands of cows vaccinated with a toxin preparation after challenge with staphylococci, while non-vaccinated cows developed a gangrenous mastitis with complete loss of udder function. Aydin and Canbazoglu (1987) vaccinated cows against *S. aureus* mastitis using a bacterin and bacterin toxoid mixture. Bacterin (with Aluminum hydroxide) elicited 100% protection for up to six months and 50% protection for eight month. The bacterin toxoid mixture elicited 50% protection up to the third and sixth months, but no protection was obtained during the eight month. Nickerson *et al.*, (1991) evaluated a commercially available bacterin that was administered systematically to 10 cows. Protein A administered in the area of the supramammary lymph node was also evaluated in 10 cows, which were boostered every 6 months and were challenged latter with *S. aureus.* After three lactation trials, there was also reduction in the number of new infections with *S. aureus* in vaccinated animals. However, the number of resolved infections was significantly higher in cows vaccinated with the protein-A vaccine or a bacterin (83 and 73 percent, respectively) compared with the non-vaccinated.

1.10 Recent specific vaccine trials against Morel's disease in the Sudan

Rodwan (1996) produced a vaccine containing capsule, whole culture and toxoid. Two doses of this vaccine (1.0 ml and 0.5 ml) given two weeks a part protected lambs challenged one months later with three times the minimum abscess causing dose (Hassan, 1996).

El Haj (2002) tested three combinations of vaccines: 60% cells with 40% toxoid; 50% cells with 50% toxoid and formalized whole culture. The culture was cultivated by the IBT bioreactor technology. Vaccinated sheep were challenged with *S. aureus* subsp. *anaerobius*. She

found the best protection when the vaccine constituted 60% cells with 40% toxoid. Also, she found that the vaccine produced by the IBT bioreactor technology was better than the vaccine produced earlier by Rodwan (1996).

1.11 The haemolytic plaque forming cell assay (PFC)

The direct plaque forming assay was initially developed by Jerne and Nordin (1963), and since then has become a mainstay of routine cellular immunology research, primarily acsessing humoral IgM antibody responses to antigen (Roitt and Delves, 1992). Briefly, B and T lineage lymphocyte populations previously presented *in vivo* with heterologous erythrocytes are agar plated in combination with the identical erythrocyte suspensions used for immunization. During incubation, the B cells secrete IgM antibody to antigenic determinants present on the surface of the erythrocytes often with T-cell help, resulting in antibody-erythrocyte binding. The presence of an adequate complement source allows complement-mediated lysis of the antibody-coated erythrocytes, resulting in the formation of clear zones or "plaques" in the agar. Located within the centre of each plaque is a single, antibody producing plasma cell. As a lytic site can be produced by a single IgM molecule fixing one C1q complement molecule, high sensitivity is a major advantage of the PFC assay (Smith *et al.*, 1999). In the Sudan PFC was tried for first time by Hassan (2001).

1.12 Opsonophagocytosis

When the pathogen enters the underlying tissues, the innate immune response components including macrophages play a primary defence role. Macrophages and other lymphocytes use toxic reactive oxygen species (ROS) such as the superoxide anion, hydrogen peroxide

and hydroxy radicals to support killing phagocytosed bacteria (Clements and Foster, 1999).

Opsonization, or enhanced attachment, refers to the antibody molecules IgG, IgE and the complement proteins C3b and C4b attaching antigens to phagocytes. This results in much more efficient phagocytosis. Optimal phagocytosis generally requires the presence of complements and specific antibodies that recognize the bacterium through Fab regions and bind the receptors on the phagocyte (Howard *et al.*, 1980). Hyperimmune serum or monoclonal antibodies directed towards surface components (e.g., capsular polysaccharide or surface protein adhesins) could theoretically prevent bacterial adherence and promote phagocytosis by opsonization of bacterial cells. Also, human hyperimmune serum could be given to hospital patients before surgery as a form of passive immunization (Kenneth Todar University, 2008).

CHAPTER TWO

MATERILAS AND METHODS

2.1 Survey

2.1.1 Collection of samples

One hundred and seventy enlarged superficial lymph nodes were taken from sheep at meat inspection in Alkadaro, Ghanawa and Alsabaloga slaughter houses. Thirty pus samples were taken from sheep affected with abscess disease in outbreak of the disease in a flock of sheep at Alsamra village, Khartoum North. Fig. 2 shows the locations of these sampling areas in the map of Khartoum State. Pus samples were collected from affected lymph nodes as follows: lymph nodes surfaces were disinfected with a piece of cotton soaked in 70% alcohol followed by hot spatula, small incisions were made using sterile blades, the pus was collected aseptically in sterile universal bottles and stored at -20 ºC until cultured. Pus from live animals was taken from incised abscess into sterile bottles after shaving and disinfection with alcohol.

2.1.2 Smears

2.1.2.1 Preparation of smears

Direct smears were prepared from pus samples in clean glass slides, dried, fixed by heating and stained.

2.1.2.2 Gram’s stain

Gram's stain was done according to the method described by Barrow and Feltham (1993).

2.1.3 Culture methods

2.1.3.1 Culturing and purification

Pus samples were streaked on 10% sheep blood agar plates and incubated in candle jars at 37 ºC for 48 h. Subcultures were made for further purification of mixed cultures.

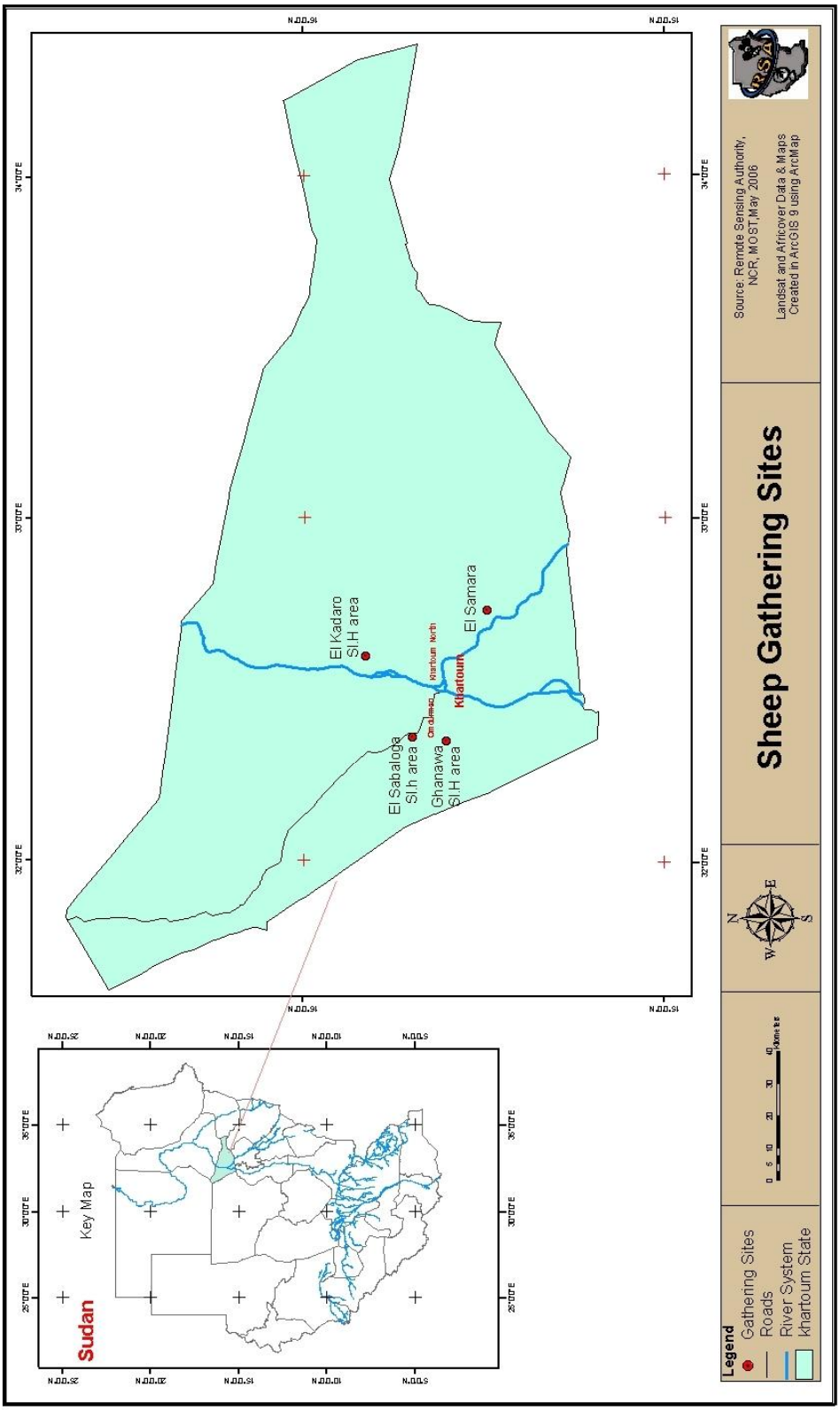


Fig. 2: Sites of sample collection

2.1.3.2. Culture media

2.1.3.2.1 Solid media

2.1.3.2.1.1 Blood Agar Base No. 2 (Oxoid), (g/l)

Proteose Peptone	15
Liver digest	2.5
Yeast extract	5
Sodium chloride	5
Agar No. 3	12

pH 7.4

Forty grams were suspended in one litre of distilled water, steamed to dissolve completely and sterilized by autoclaving at 121 ºC for 15 min. Defibrinated sheep blood was added to a final concentration of 10% after the agar being cooled to 50 ºC, mixed gently and dispensed onto sterile Petri-dishes.

2.1.3.2.1.2 Nutrient agar

Twenty eight grams of nutrient agar (Oxoid CMR_{32}) were dissolved in 1000 ml of distilled water, mixed and sterilized by autoclaving at 121˚C for 15 min. The medium was then poured into sterile universal bottles and set in a slope position to solidify.

2.1.3.2.1.3 Urea agar base (g/l)

Peptone	1
Dextrose	1
Sodium Chloride	5
Disodium Phosphate	1.2
Potasium Dihydrogen Phosphate	0.8
Phenol Red	0.012
Agar no. 3	15g

pH 6.8

An amount of 2.4 g of urea agar base were suspended in 95 ml of distilled water, steamed to dissolve completely, sterilized by autoclaving at 115 ºC for 20 min and added aseptically to 5 ml of 40% urea solution after cooling to 45 ºC. After being mixed well, the medium was distributed into 10 ml sterile MacCarteny bottles and set in a slope position to solidify.

2.1.3.2.1.4 Milk agar

Oxoid skimmed milk L31 was used. A volume of 50 ml of 10% of milk solution was added to 100 ml of nutrient agar, mixed well, sterilized at 110 ˚C for 5 min, cooled to 50 ˚C and distributed onto sterile Petri-dishes in portions of 15 ml each.

2.1.3.2.2 Liquid medium

2.1.3.2.2.1 Nutrient Broth

Thirteen grams of nutrient broth (Oxoid M1) were added to 1000 ml of distilled water. The medium was distributed into 10 ml in universal bottles and then sterilized by autoclaving at 121 ºC for 15 min.

2.1.3.2.2.2 Brain Heart Infusion (g/l)

Calf brain infusion solid	12.5
Beef heart infusion	5.0
Proteose peptone	10.0
Glucose	2.0
Sodium chloride	5.0
Di-Sodium phosphate	2.5

pH 7.4

Thirty seven grams were suspended in one litre of distilled water, boiled to dissolve completely and distributed into 20 ml bottles and sterilized by autoclaving at 120 ˚C for 15 min.

2.1.3.2.2.3 Peptone water (Oxoid) (g\l)

Peptone	10
Sodium chloride	5

pH 7.2

Fifteen grams were added to one litre of distilled water, mixed well, distributed into sterile test tubes and autoclaved at 121 ˚C for 15 min.

2.1.3.2.2.4 MR-VP medium (Glucose phosphate medium)

Five grams of peptone and five grams of K_2HPO_4 were dissolved in 1000 ml of distilled water by steaming and filtered; the pH was adjusted to 7.5. Five grams of glucose were added; the medium was then distributed in 1.5 ml volumes into test tubes and sterilized by autoclaving at 110 ºC for 10 min.

2.1.3.2.2.5 Peptone water sugars

Peptone water	900 ml
Andrade's indicator	10 ml

The pH of the peptone water was adjusted to 7.1 - 7.3 before adding the Andrade's indicator. Ten grams of the appropriate sugar dissolved in 90 ml of distilled water were added to the mixture and mixed thoroughly, distributed in five ml portions into sterile test tubes and sterilized by autoclaving at 110 ˚C for 10 min.

2.1.3.2.2.6 Nitrate broth

KNO_3 was dissolved in the broth, distributed in sterile test tubes, and sterilized by autoclaving at 115 ˚C for 10 min.

2.1.3.6 Biochemical tests

2.1.3.6.1 Aerobic growth

Test organisms were inoculated on blood agar plates, incubated aerobically at 37 ˚C for 48 h and then checked for growth.

2.1.3.6.2 Haemolytic activity

Blood agar plates were inoculated with the test organisms, incubated at 37 ˚C for 48 h, put at 4 ˚C for 24 h. The haemolysis was read thereafter to perform hot cold haemolysis test.

2.1.3.6.3 Catalase test

A drop of 3% aqueous solution of hydrogen peroxide (H_2O_2) was placed on a clean microscopic slide, then with glass or wood loop a colony of the test organism was added to it. The test is considered positive when gas bubbles appear on the surface.

2.1.3.6.4 Oxidase test

Strips of filter papers soaked in 1% solution of tetramethylene-p-phenylene diamine dihydrochloride were used in this test. Young colonies were picked with sterile bent glass rods and rubbed on the filter paper. Reactions were considered positive when dark purple colour appeared within 5-10 seconds.

2.1.3.6.5 Colony size and colour

Milk agar plates containing fresh colonies were placed on the bench for overnight at room temperature. The size of a single colony was measured, and the colour was noticed.

2.1.3.6.6 Coagulase slide test

To detect bound coagulase (clumping factor), a drop of physiological saline was placed on a slide. A few colonies were emulsified in the drop to make a thick suspension. A drop of undiluted human plasma was placed at the end of the slide and then mixed gently with the bacterial suspension. Clumping of the organism within 10 seconds was considered as a positive reaction.

2.1.3.6.7 Coagulase test

Equal amounts of diluted human plasma (1% in normal saline) and

48 h culture in nutrient broth were mixed carefully, and examined after 1, 2 and 6 hours. Negative tubes were further incubated overnight and then re-examined.

2.1.3.6.8 Sugar fermentation test

The ability to ferment seven sugars was tested. The sugars were mannitol, maltose, lactose, xylose, trehalose, fructose, mannose, raffinose and sucrose. Each isolate was inoculated in a set of the seven sugars. The tubes were then incubated and examined for up to 7 days. Change of colour to pink indicated positive results.

2.1.3.6.9 Urease test

Urea agar slope was heavily inoculated with the test culture and then incubated at 37 ˚C for 48 h. Positive reaction was indicated by change of colour of the medium to the pinkish.

2.1.3.6.10 Novobiocin sensitivity test

The test organisms were spread on the surface of the blood agar using a swab. Discs containing 5 µg of novobiocin were applied on the plates using sterile forceps. The plates were incubated at 37 ˚C for 24 h. Positive results showed a clear zone around the discs.

2.1.3.6.11 ß-Galactosidase test

A loop full of colonies was streaked on a filter paper placed into a plate and 20 µl of ß-Galactasidase were added to it. The plate was covered with aluminium foil and incubated at 37 °C for 1 h. A volume of 20 µl of NaOH was added before being read under UV light. Positive results were indicated by the development of a fluorescent blue colour.

2.2 Molecular techniques for characterization of *S. aureus* subsp. *anaerobius* isolates

Molecular biology techniques were used to confirm and to compare the local isolates with the reference strains. Representative isolates were

randomly selected for these tests.

2.2.1. TBE Buffer (Tris-Borate-EDTA) 10x (pH 8.3)

Tris base	108 g
Boric acid	55 g
Na_4EDTA	9.3 g
ddH_2O	1000 ml

pH 8.3 (without adjustment).

2.2.2 PCR Master Mix

Super Hot Master Mix of Bioron (Bioron, Ludwigshafen, Germany). This mixture contained: Taq DNA polymerase in reaction buffer 0.1 unit/µl, antibodies to Taq DNA Polymerase, (NH4)2SO4 32 mM, Tris HCl, pH 8.8 at 25 °C, 130 mM, 0.2% Tween 20, MgCl2 3mM and dNTP (dATP, dCTP, dGTP, dTTP) 0.4 mM of each.

2.2.3 Agarose gel (2%)

For small size gels, 2.4g of agarose were added to 120 ml of 1x TBE buffer; heated in microwave to melt, mixed well before addition of 50 µl of ethidium bromide; the mixture was poured into the appropriate plate and left to solidify after insertion of the appropriate comb.

2.2.4 Isolates for the molecular characterization

Twenty two isolates of *S. aureus* subsp. *anaerobius* (Table 1) obtained from lymph node abscesses of sheep at different 3 areas (7 from outbreak of sheep abscess in Alsamra village, 9 from Alkadaro slaughter house and 6 from Ghanawa slaughter house).

2.2.5 DNA extraction

Genomic DNA was extracted using Axy Prep Bacterial Genomic DNA Miniprep Kit of Axygen (Bioron, Ludwigshafen, Germany) with some modifications of the manufacturer's protocol:

- 3-5 colonies of *S. aureus* subsp. *anaerobius* obtained from 48 h blood

agar culture were suspended in 150 µl of the recommended buffer (after adding the RNase).

- 10 µl of Lysostaphin (Sigma, Taufkirchen, Germany) 1mg/ml were added to the mixture and incubated at 37 °C for 1 h.
- 2 µl of 10% Proteinase K (Bioron) was added and incubated at 56 °C for 2 h.

2.2.6 DNA concentration

For all samples, the DNA concentration was measured by a spectrophotometer (Nanodrop ND1000, Peqlab, Erlangen, Germany).

2.2.7 Purification of the PCR products for sequencing

Montage PCR Centrifugal Filter Devices (Millipore, Bedford, USA) were used to purify PCR products prior to sequencing.

2.2.8 Primers

Primers used in this part are listed in table (2). All primers were synthesised by ThermoFisher Scientific, Germany (Thermo Electron, Oberhausen, Germany).

2.2.9 PCR reaction mixture

For 25 µl PCR reaction mixture, the following were mixed:

Master Mix	12.5 µ
Primer 1	0.5 µ
Primer 2	0.5 µ
ddH_2O	11.5 µ

A volume of 23 µl of the PCR mixture was added to 2 µ of the DNA template.

Table 1: *Staphylococcus aureus* subsp. *anaerobius* used in this study

No.	Code	Source
1	K1	Alkadaro slaughterhouse
2	K8	Alkadaro slaughterhouse
3	K10	Alkadaro slaughterhouse
4	K11	Alkadaro slaughterhouse
5	K17	Alkadaro slaughterhouse
6	K18	Alkadaro slaughterhouse
7	K22	Alkadaro slaughterhouse
8	K41	Alkadaro slaughterhouse
9	G2	Ghanawa slaughterhouse
10	G35	Ghanawa slaughterhouse
11	G40	Ghanawa slaughterhouse
12	G42	Ghanawa slaughterhouse
13	G58	Ghanawa slaughterhouse
14	G97	Ghanawa slaughterhouse
15	S7	outbreak in Alsamra village
16	S8	outbreak in Alsamra village
17	S9	outbreak in Alsamra village
18	S10	outbreak in Alsamra village
19	S14	outbreak in Alsamra village
20	S16	outbreak in Alsamra village
21	S18	outbreak in Alsamra village
22	S19	outbreak in Alsamra village
23	Reference strain	ATCC35844, DSM no. 20714*
24	Reference strain	IBT Culture Collection Göttingen no.9199/2628

*DSM: Deutsche Sammlung von Microorganismen und Zelkulturen, Braunschweig, Germany.

Table 2: Oligonucleotides used in this study

Primer name	Sequence	Reference
nuc 1	5´GCGATTGATGGTGATACGGTT 3´	Brakstad *et al*. (1992)
nuc 2	5´AGCCAAGCCTTGACGAACTAAAGC 3´	Brakstad *et al*. (1992)
3 F	5´ GCTTTTTAAGTGTACTATTC 3´	This study
164 F	5´ TATAAATTGTGGAGGGATGAC 3´	Sanz *et al.* (2000)
808 F	5´ CTCCATTTTAGAACGCAACAA 3´	Sanz *et al*. (2000)
1396 F	5´ GATGGATACGGCTATGAATA 3´	This study
872 R	5´ GCTATAATTTCAGCAGCTTC 3´	This study
1583 R	5´ TGGGTCAGCTTTGTAACA 3´	Sanz *et al.* (2000)
1726 R	5´ TCATAAACTGCTCAACTACGC 3´	Sanz *et al.* (2000)
786	5´GCGATCCCCA 3´	Pereira *etal.*(2002)
798	5´ TGACCCGCC 3´	Pereira *etal.*(2002)
spa 1	5´ CAAGCACCAAAAGAGGAA 3´	Frénay *et al.* (1994)
spa 2	5´ CACCAGGTTTAACGACAT 3´	Frénay *et al.* (1994)
coa 1	5´ CGAGACCAAGATTCAACAAG 3´	Goh *et al.* (1992)
coa 2	5´ AAAGAAAACCACTCACATCA 3´	Goh *et al*. (1992)

2.2.10 PCR reaction conditions

PCR reaction conditions used for the amplification of different genes targeted in this study are shown in Table 3.

Table 3: PCR thermocycler protocols used in this study

Step/	Nuclease		Catalase		RAPD		Polymorphism	
Protocol	°C	min	°C	min	°C	min	°C	min
First	94	5	94	10	-	-	-	-
Denaturation	94	1	94	1	94	1	95	0.5
Annealing	55	0.5	52	1	36	1	55	2
Extension	72	1.5	72	1.5	72	2	72	4
Final	72	3.5	72	10	72	7	72	5
Cycles	37		30		30		40	

2.2.11 Gel documentation

PCR-amplification products were subjected to electrophoresis in agarose gel (section 2.2.2) in lx TBE buffer using horizontal electrophoresis system (Power *N* PAC 3000, BioRad, Munich, Germany). PCR products (18 or 9 µl) were mixed with 1 or 2 µl of bromphenol blue stain before being applied to wells. Seven µl of the molecular weight standard (λ DNA-Hind III/ΦXHae III (Finnzymes, Espoo, Finland) was included in each gel. One hundred constant volts were applied to the gels for 1 h. DNA amplified segments were visualized by UV illuminator (MWG, Ebersberg, Germany) connected to a PC and a monitor.

2.2.12 *nuc* gene detection

PCR amplification of the nuclease (*nuc*) gene was done so as to confirm the biochemical identification of the isolates as *S. aureus*. The primers designed by Brakstad *et al*. (1992) were used for this purpose (Table 2).

2.2.13 Catalase gene (*kat* gene)

2.2.13.1 Amplification of the catalase gene (*kat* gene)

Many segments of the catalase gene were amplified using many sets of primers (Table 2). The primers were designed in this study based on the sequences of the catalase genes of *S. aureus* strain MVF213 (GenBank accession no. AJ000471) and *S. aureus* strain ATCC12600 (GenBank accession no. AJ000472).

2.2.13.2 Sequencing of the catalase gene

PCR amplification products of some segments of the catalase gene were sequenced in this part. Sequencing of the whole catalase gene of isolate S10 was achieved by partial sequencing using sets of primers as follows: 808F and 1583R; 164F and 1396R; 1396F and 1726 R; 3F and 872R. Partial sequencing of about 990 bp of other five (K22, K41, G2, G35 and S19), and about 700 bp of three (K1, G97 and S7) local isolates in addition to one reference strain (ATCC35844, DSM no. 20714) was done using primer 1583R.

Purified PCR products plus the diluted primers (10 pM) were sent to in a commercial company (SeqLab, Göttingen, Germany) in an ABI sequencer.

2.2.13.3 Sequence alignment and editing

The sequences of the PCR products were edited by BioEdit Sequence Alignment Editor, version 7.05.3 (10/28/05).

2.2.14 RAPD-PCR

Randomly amplified polymorphic DNA PCR (RAPD) was used to detect possible differences between the strains. Two primers (786 and 798) designed by Pereira *et al.* (2002) were used (Table 2). Amplification conditions are shown in Table 3.

2.2.14.1 RAPD optimization (Confirmatory test for $MgCl_2$)

When the PCR reaction mixture mentioned by Pereira *et al.* (2002) was used, no clear amplicons were seen. So, two additional $MgCl_2$ concentrations (4.5 mM, and 6 mM) in the PCR reaction mixture were evaluated.

2.2.15 Pulsed-field gel electrophoresis (PFGE)

2.2.15.1 Buffers

2.2.15.1.1 Lysis buffer, pH 7.6

Tris	6 mM
NaCl	1 mM
EDTA	10 mM.
Brij-58	5 g.
Sodium deoxycholate	2 g.
Sodium lauroylsarcosine	5 g.
Lysozyme	5 g.
Lysostaphin	5000 units
dd H_2O	1000 ml

2.2.15.1.2 Washing buffer (Tris - EDTA), pH 8.0

TrisHCl	10 mM
EDTA	1 mM
dd H_2O	1000 ml

2.2.15.2 PFGE Protocol

-Overnight cultures of bacterial cells were harvested and resuspended in 10 mM Tris-EDTA after washing in TE buffer (l0 mM Tris-HCl, 50 mM EDTA; pH 7.5).

- The bacterial suspension was adjusted to a concentration of $1x10^9$ cfu/ml by using Mc Farland tube No. 4 turbidity.

-200 µl of the bacterial suspension was added to an equal volume of 2% low-melting point agarose, 6 µl of lysostaphin were added and mixed well before being allowed to solidify in a plug mold (Bio-Rad).

-Each agarose block was removed from the mold and suspended in the lysis solution. The bacterial cells were lysed by subsequent incubation of the blocks in 100 µl lysis buffer at 37 °C for 6 h.

-The gel plugs were incubated overnight at 55 °C in 2 ml of proteinase K (10 mg/ ml), with gentle shaking.

-The plugs were washed 3 times with a cold TE buffer for 20 minutes. Slices of the plug were cut and digested with 40 U of restriction endonuclease *Sma*I (Bioron) in the recommended restriction enzyme buffer (supplied by the manufacturer) and incubated overnight at 30 °C.

-The plugs were then loaded into 1% agarose gel wells, and sealed with 2% low-melting point agarose.

-The contourclamped homogeneous electric field apparatus from Bio-Rad was used to separate the DNA fragments.

-The gel was subjected to electrophoresis for 24 h at 15 °C with a voltage of 175 V and pulse times of 15 to 30 s in 0.05 M Tris-borate-EDTA buffer.

-The pulse times used were 5 s to 15 s for 8 h followed by 15 s to 25 s for 10 h.

-The gel was then visualized and photographed using a UV Trans-illuminator (MWG, Esberg, Germany) connected to a PC and a monitor.

2.3 Animal experiments

2.3.1 Pathogenecity of *S. aureus* subsp. *anaerobius*

Blood agar culture of *S. aureus* subsp. *anaerobius* was inoculated in brain heart infusion broth and incubated at 37 °C for 48 h. The culture was counted according to Miles and Misra (1938), and different dilutions were made.

Each of five Hamari lambs (about 10 months old) was inoculated with six different doses of the organism as shown in Table 4. The inoculums were injected subcutaneously after shaving and proper disinfection.

Table 4: Number of the inoculated organisms per lamb for pathogenecity test

Dose no.	No. of organisms (CFU)	Site of inoculation
1	480	Right (upper neck)
2	2400	Right (middle neck)
3	4800	Right (lower neck)
4	24000	Left (upper neck)
5	48000	Left (middle neck)
6	480000	Left (lower neck)

2.3.2 Pathogenecity of other staphylococci

I) Five 8-12 months old Hamary male sheep were kept for 15 days as adaptation period, during which they were given doses of anthelmentics and antibiotics. Each of five different *Staphylococcus* species (*S. aureus* subsp. *anaerobius*, *S. aureus*, *S. caseolyticus*, *S. lugdenensis* and *S. simians*), which were isolated in this study, was inoculated in one animal. Blood agar cultures of the organisms were inoculated into Brain Heart

Infusion broth, incubated at 37 °C for 48 h before being inoculated to the animals. Each animal was noculated with 1200 cfu of each organism in right middle of the neck after shaving, cleaning with cotton soaked in 70% alcohol and drying with sterile gauze.

II) Each of other four animals, prepared as mentioned above, was inoculated using one mixture of two isolates:

-*S. aureus* subsp. *anaerobius* + *S. aureus*

-*S. aureus* subsp. *anaerobius* +*S. caseolyticus*,

-*S. aureus* subsp. *anaerobius* +*S. lugdenensis*

-*S. aureus* subsp. *anaerobius* + *S. simians*

A fifth ram was inoculated with *S. aureus* subsp. *anaerobius* alone as control.

2.3.3 Vaccination and challenge

2.3.3.1 The vaccine

The vaccine used in this study was originally prepared by Rodwan (1996). It was prepared from broth cultures of *S. aureus* subsp. *anaerobius* supplemented with 2% horse serum. The cultures were incubated under anaerobic conditions, at 37 °C for 48-72 h. The media were constantly adjusted to pH 7.2.

2.3.3.1.2 Ingredients of the vaccine

The vaccine consisted of the following mixtures of antigens:

(i) Whole formalinized culture.

(ii) Toxoid.

(iii) Capsule.

These components were prepared by the following procedures:

(i) Toxoid

The supernatant of broth cultures (on brain heart infusion broth and RCM) of *S. aureus* subsp. *anaerobius* (Isolate no. 11) were filtered

through a 0.4 µm Seitz filter and then concentrated twice using polyethylene glycol; pH of the concentrated toxin was adjusted to 7.0; formaline was added to a final concentration of 0.5% (v/v) to inactivate the toxin.

(ii) Capsular antigen

The capsular antigen was prepared by culturing *S. aureus* subsp. *anaerobius* on a special solid medium containing peptone, beef and yeast extracts, Na, Mn, K, salts in addition to horse serum, glucose and charcoal according to Rajab (1997).

The vaccine strain of the organism was cultured on this medium, incubated in 10% CO_2 tension at 37 °C for 24 h. Cultures were subsequently washed with 4 ml of 1% phenol and then kept at 4 °C for at least two weeks before use.

2.3.3.1.3 Mixing different ingredients of the vaccine

Thirty millilitres of the double concentrated toxoid were added to 45 ml of formalinized 48 h culture in brain heart infusion broth, pH of the mixture was adjusted to pH 7.0, 25 ml of the medium containing capsule were added. All ingredients were added while gentlely shaking; the pH was maintained at pH 7.0. Formaldehyde was added to a final concentration of 0.5 % v/v. The final vaccine was further diluted by addition of 75% of the mixture and 25% sterile normal saline.

2.3.3.2 Evaluation of the effective dose of the vaccine

2.3.3.2.1 Titration of the vaccine

Eighteen, 8-12 months old, Hamari male sheep were purchased from the local market. The animals were kept for adaptation period, during which they were given prophylactic doses of anthilmentics, and antibiotics and were sprayed with acaricides. The animals were divided into 6 groups. Each one of the 5 groups received a different dose of the

vaccine, while the sixth group served as a control non-vaccinated. The vaccine was diluted with sterile normal saline and completed to one millilitre as shown in Table 5. The doses were 0.25, 0.75, 0.5, 1 and 0.5 m. The last group received a booster dose of another 0.25 ml after 2 weeks. The vaccine doses were injected subcutaeonusly at the right side in the middle crest of the neck after shaving. The sites of injection were examined daily for up to 21 days and the rectal temperature was recorded, whole blood for haemogram was collected weekly. Injection sites of the vaccine and challenge were examined daily and palpated for post-vaccinal tissue reaction.

2.3.3.2.2 Challenge

After 21 days, animals of all groups (the previous section) were challenged with 1200 cfu of *S. aureus* subsp. *anaerobius* as in section 2.3.1. The sites of inoculation were examined daily for up to 14 days for development of abscesses. Rectal temperature was recorded. Whole blood for haemogram was collected weekly. The diameter of the inoculation sites were recorded in mm. Pus samples were collected from the discharging abscesses in sterile containers and cultured in the same day on blood agar.

Table 5: Vaccination trials of groups of sheep with different doses of the vaccine against Morel's disease

Group	A	B	C	D	E		Control
					initial	booster	
Dose of vaccine (ml)	0.25	0.50	0.75	1.00	0.50	0.25	0
Diluent (Sterile Normal Saline), ml	0.75	0.50	0.25	0	0.50	0.75	1.0
Total (ml)	1.0	1.0	1.0	1.0	1.0	1.0	1.0

2.3.3.2.2 Evaluation of the vaccine against different staphylococci

This experiment was conducted to evaluate the ability of Morel's disease vaccine to protect against abscess formation caused other staphylococci.

2.3.3.2.2.1 Vaccination and challenge with one *Staphylococcus* species

Five, 8-12 months old male Hamary sheep were kept for adaptation period of one week after receiving prophylactic doses of anthilmentic, antibiotic and being sprayed with acaricides. The animals were vaccinated with 0.5 ml of the vaccine and boostered after 15 days with 0.25 ml.

The sheep were challenged after one month from the first dose of the vaccine with 4,800 cfu of one species of staphylococci isolated in this study. Animals were daily examined for up to 21 days.

2.3.3.2.2.2 Vaccination and challenge with two *Staphylococcus* species

Ten 8-12 months old Hamary male sheep were kept for a period of adaptation of one week after being given prophylactic doses of anthilmentic, antibiotic and sprayed with acaricide. All animals were vaccinated with 0.5 ml of the vaccine and boostered after 15 days using 0.25 ml. The animals were divided into two groups; each group was challenged- one month after vaccination- with a mixture of *S. aureus* subsp. *anaerobius* (1200 cfu) and 0.5 of neat culture (about 2,400,000 cfu) of either of *S. aureus* and *S. caseolyticus*. Two animals of each group received one species as control.

2.3.3.2.2.3 Post-mortem examination

All animals were slaughtered three weeks after challenge. Macroscopic findings were recorded in control sheets. Samples were taken for both bacteriology and histopathology in addition to impression smears. One spleen from each group was taken for plaque forming cell

assay test.

2.6 Immunological tests

2.6.1 Plaque forming cell assay

2.6.1.1 Preparation of the antigen

Sheep blood was collected in Alsever's solution (10%). The red blood cells (RBCs) were washed 3 times with phosphate buffer saline (PBS), pH 7.4. Equivalent volumes of 10% RBCs and tannic acid solution (5 mg tannic acid in 100 ml PBS) were mixed and incubated at 37 °C for 20 min. The Ag (vaccine) was dissolved in PBS pH 6.4 at concentration of 0.2-mg/ ml. Equivalent volumes of Ag, with tanned sheep RBCs were mixed well and incubated at 37°C for 20 minutes. The combination was then washed three times in normal saline containing 0.5% heat inactivated normal rabbit serum (at 56°C).

2.6.1.1.2 Sheep red blood cells (SRBCs)

Sheep blood was washed twice in Phosphate Buffered Saline (PBS) and once in Balanced Salt Solution (BSS)- "Hank's Solution"-, then the blood was diluted to 1 in 3 with BSS. RBCs were washed three times with PBS and then re-suspended as 20% volumes.

2.6.1.1.3 Effector cells

The splenic cells were collected from the four groups of sheep: before vaccination, one week after vaccination, two weeks after vaccination and one week after the booster dose of the vaccine. The cells were washed three times in BSS and re-suspended as 10% in BSS

2.6.1.1.4 Agarose

The agarose was dissolved in BSS at 0.5% in a 100 °C water bath and then held at 47 °C until used.

2.6.1.1. 5 Complement

Freeze-dried Guinea pig serum (Wellcome, UK) was used as

complement source. The contents were reconstituted in 2 ml sterile distilled water and then diluted to 1 in 3 with BSS.

2.6.1.1.6 Balanced Salt Solution (BSS)

The following ingredients were dissolved:

Phenol	10 mg
$CaCl_2$	140 mg
NaCl	800 mg
KCl	100 mg
$MgSO_4.7H_2O$	200 mg
$MgCl_2.6H_2O$	200 mg

The salts were added to one litre of distilled water, the pH was adjusted to 7.0-7.2 and the solution was stored at -20 °C until used.

2.6.1.2 Plaque forming cell assay mixture

Small test tubes were placed in 47 °C water bath; to each tube the following items were added:

Agarose	300 µl
RBCs	20 µl
Spleen cell suspension	100 µl
Complement (dil. 1:3, added while whirl mixing)	40 µl

All components were mixed rapidly on a whirl-mixer, poured on a slide and allowed to set. The slides were incubated at 37 °C in humidity chamber, examined after one hour by the naked eye and low power lens of the microscope (10x) and also after overnight incubation.

2.6.1.2 Validity of spleen cells

Viable spleen cells count was done every day untill all cells were dead, using trypan blue staining method. The cells were stored in either RPMI; Histopaque solution or were left without storage solution.

2.6.2 Opsonophagcytosis tests

Phagocytosis of *S. aureus* by ovine polymorphnuclear cells (neutrophils) in the presence or absence of opsonin was measured using a modification of the method of Verhoef *et al*. (1977) as below.

2.6.2.1 Bacterial growth

S. aureus subsp. *anaerobius* were grown in 5 ml nutrient broth at 37 °C for 48 h and used at a concentration equivalent to McFarland's opacity tube No. 4, which is equivalent to 1.2 X 10^9 cell/ml, according to Baron *et al*. (1994).

2.6.2.2 Blood samples

Fresh sheep blood was collected from the jugular vein of healthy sheep using sodium citrate as anticoagulant. A volume of 0.9 ml of sterile PBS was pipetted into each of four test tubes under aseptic conditions; 0.1 ml *S. aureus* subsp. *anaerobius* culture was added to the first tube and serially diluted. Dilution 1/1000 was used in this test.

2.6.2.3 Opsonization method

The test was performed in three eppendorf tubes. In the first tube 0.2 ml of the diluted culture of *S. aureus* subsp. *anaerobius* was added to 0.1 ml of the vaccinated sheep serum; in the second eppendorf tube 0.2 ml of the diluted culture of *S. aureus* and 0.1 ml normal sheep serum; the third and fourth eppendorf tubes served as controls containing 0.2 ml of diluted cultures (*S. aureus* or *S. aureus* subsp. *anaerobius*) and 0.6 ml sheep blood. The tubes were incubated for 30 min with mixing every 10 min. After incubation, 0.5 ml of fresh sheep blood was added to each tube.

Phagocytosis assay was carried out by incubation of the eppendorf tubes at 37 °C with frequent mixing for 1 h. One ml of the mixture of each tube was then inoculated subcutaneously into experimental animals

(Hamary sheep) at 0 and 120 min and spread at the same time on blood agar plates by dropping 10 µl into blood agar plates. Each experiment was done in duplicates. The plates were incubated at 37 °C for 24 h under 10% increased CO_2 tension, growing colonies were counted.

CHAPTER THREE

RESULTS

3.1 Survey for sheep abscess disease

3.1.1 Isolates from lymph nodes of animals at meat inspection

From 170 pus samples of infected lymph nodes collected from sheep at meat inspection at Alkadaro, Ghanawa and Alsabaloga slaughter houses, 117 (68.8%) were *Staphylococcus* spp., 45 (26.5%) *Corynebacterium* spp. and 8 samples (4.7%) yielded both *Staphylococcus* spp. and *Corynebacterium* spp. Fig. 3.

Staphylococcus aureus subsp. *anaerobius* was the most prevalent among staphylococci isolates (63.2%) followed by *S. caseolyticus* (21.3%), *S. aureus* (11.9%) and *S. simians*, *S. lugdunensis*, *S. warneri*, *S. epidermidis* (each 0.9%) as shown in Fig. 4 and Table 6.

3.1.2 Isolates from outbreak of sheep abscess disease

The outbreak occurred in a flock of sheep in Alsamra village in Khartoum State. The animals were freely raised in natural grazing area during the day and they used to reside to pen in the evenings, where they received some type of feed supplementation. The morbidity among herd was 30%. Two females were infected and lambs of about 2 months of age were also infected. The commonly infected lymph nodes were prescapular, parotid and submandibular (Fig. 5). From 28 animals (93.3%) of the pus samples yielded pure cultures of *S. aureus* subsp. *anaerobius*, while the rest two animals (6.7%) yielded *Corynebacterium* spp.

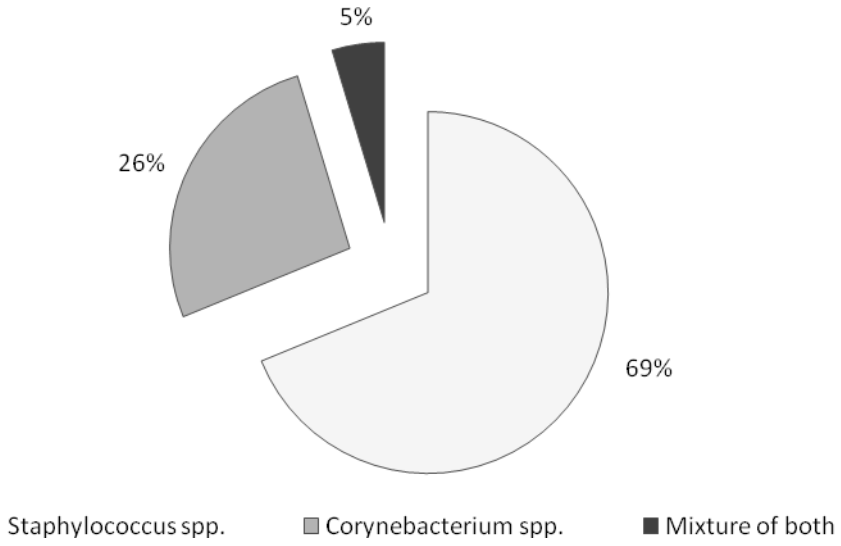


Fig. 3: Bacteria isolated from superficial lymph abscess of sheep at meat inspection

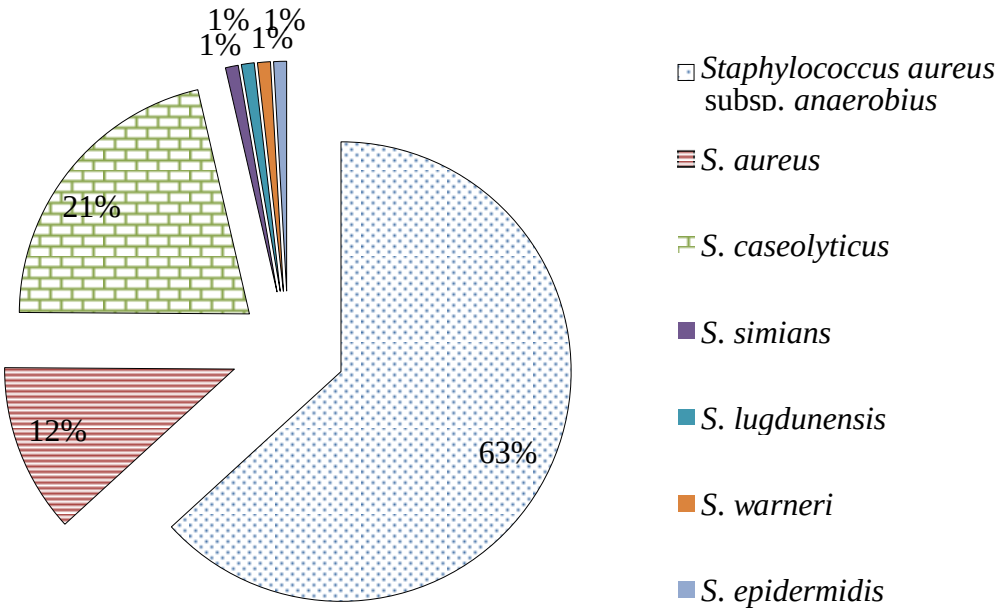


Fig. 4: *Staphylococcus* spp. isolated from superficial lymph node abscesses of sheep at meat inspection

Fig. 5: Sheep flock in Alsamra village, Khartoum North, Sudan, in which outbreak of abscess disease occurred. A, C, abscesses in the parotid lymph node; B, abscesses in the parotid and submandibular lymph nodes; D, abscesses in the parotid and prescapular lymph nodes; E: part of the flock in natural grazing area.

Table 6: *Staphylococcus* species isolated from infected superficial lymph nodes anscesses of sheep at meat inspection in Alkadaro, Ghanawa and Alsabaloga slaughter houses in Khartoum State

No.	*Staphylococcus* spp.	Total no.	%
1	*S. aureus* subsp. *anaerobius*	74	63.2
2	*S. caseolyticus**	25	21.3
3	*S. aureus*	14	11.9
4	*S. simians*	1	0.9
5	*S. lugdunensis*	1	0.9
6	*S. warneri*	1	0.9
7	*S. epidermidis*	1	0.9

* *Staphylococcus caseolyticus* has been renamed *Macrococcus caseolyticus*

3.2 Properties of staphylococci isolated from sheep abscesses

All isolates grew well under anaerobic conditions after 48 h incubation at 37 °C. *S. aureus* subsp. *anaerobius* colonies were smooth, glistening, convex, about 1 mm in diameter (Fig. 6). Colony properties of other staphylococci are shown in Table 7.

Table 7: Colonial morphology of staphylococci isolated from superficial lymoh node abscesses of sheep

No.	*Staphylococcus* spp.	Total no.	%
1	*S. aureus* subsp. *anaerobius*	74	63.2
2	*S. caseolyticus**	25	21.3
3	*S. aureus*	14	11.9
4	*S. simians*	1	0.9
5	*S. lugdunensis*	1	0.9
6	*S. warneri*	1	0.9
7	*S. epidermidis*	1	0.9

* *Staphylococcus caseolyticus* has been renamed *Macrococcus caseolyticus*

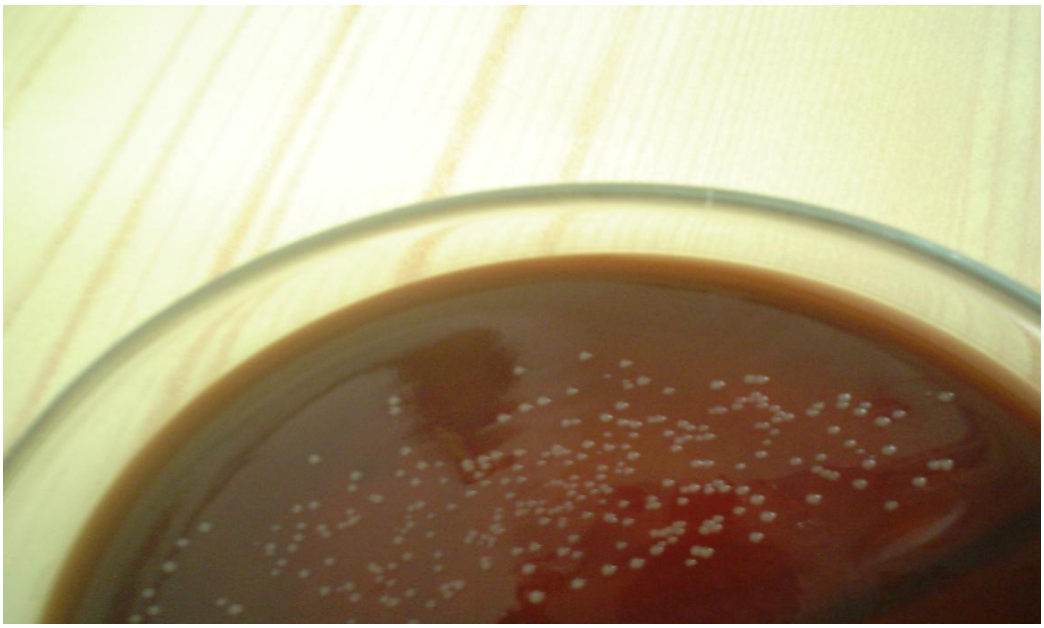


Fig 6: *Staphylococcus aureus* subsp. *anaerobius* colonies grown on blood agar medium.

3.3 Biochemical properties

The biochemical properties of bacteria isolated in this study are shown in Tables 8 and 9.

All *S. aureus* subsp. *anaerobius* isolates were anaerobic, 95% grew as pin point colonies after 4-5 days of aerobic incubation, all were haemolytic; catalase, oxidase, manniol, and B-galactosidase negative; all were positive for the clumping factor and coagulase.

Table 8: Biochemical properties of staphylococci isolated from lymph node abscesses of sheep at meat inspection and with Morel's Disease

Table 8: continued

Table 8: continued

Table 9: Biochemical properties of *Staphylococcus aureus* subsp. *anaerobius* isolated in this study

No.	Test	No. of positive isolates	No. of negative isolates	No. of doubtful isolates
1	Anaerobic growth	74	-	-
2	Aerobic growth[1]	-	4	70
3	Haemolysis[2]	74	-	-
4	Oxidase	-	74	-
5	Catalase	74	-	-
6	Coagulase[3]	74	-	
7	Mannitol (anaerobic)	-	74	-
8	ß- galactosidase	-	74	-
9	Clumping factor	74	-	-
10	VP	-	72	2

1: very small colonies after 4-5 days, 2: double zoon, hot cold haemolysis, 3: after 1 hour and also after 24 overnight incubation.

3.9 Molecular biology results

3.9.1 DNA concentrations

DNA concentrations extracted from the isolates used in molecular characterization are shown in Table 12.

Table 10: DNA concentrations of *S. aureus* subsp. *anaerobius* isolates used in the part of molecular characterization

No.	Isolate code	DNA concentration (ng/µl)
1	K1	3.6
2	K8	6.3
3	K10	2.3
4	K11	2.9
5	K17	2.1
6	K18	1.6
7	K22	1.1
8	K41	0.7
9	G2	0.8
10	G35	1.2
11	G40	1.4
12	G42	2.3
13	G58	2.4
14	G97	1.4
15	S7	2.7
16	S8	3.1
17	S9	3.0
18	S10	3.0
19	S14	7.3
20	S16	2.3
21	S18	8.9
22	S19	15.5
23	Reference strain ATCC35844, DSM no. 20714	4.2
24	Reference strain IBT-Göttingen Culture Collection No. 9199/2628	1.3

DSM: Deutsche Sammlung von Zellkulturen und Mikroorganismen

3.9.2 *Nuc* gene detection

All *S. aureus* subsp. *anaerobius* isolates yielded amplicons of the *nuc* gene as shown in Fig.7.

3.9.3 Catalase gene (*kat* gene)

3.9.3.1 Detection of the catalase gene

All *S. aureus* subsp. *anaerobius* isolates yielded positive amplification results of the catalase gene. Amplicons of the catalase gene using different sets of primers are shown in Figs. 8, 9 and 10.

3.9.3.2 Sequencing results of the catalase gene

The complete sequence of the catalase gene of S10, isolated from the outbreak in Alsamra village, is shown in Table 11 and Appendix 1.

The whole amplified part of the putative catalase gene of strain S10 (*katS10*) was 1725 nucleotides in length. The open reading frame starts at base 164 (ATG, the initiation codon), and ends at base 1999 (TGA, the stop codon). This sequence was deposited in the GenBank under accession no. EU281993 (Appendix 2).

Catalase gene sequence of *S. aureus* subsp. *anaerobius* strain S10 (outbreak isolate) showed 99% identity to that of *S. aureus* subsp. *anaerobius* MVF213 (GenBank accession no. AJ000471), *S. aureus* subsp. *aureus* NCTC8325, *S. aureus* subsp. *aureus* strain Newman (GenBank accession nos.CP000253 and AP00935.1, respectively) as shown in Appendices 2 and 3.

Comparison of this sequence with the sequence of *katB* (the catalase-like protein of *S. aureus* subsp. *anaerobius* MVF214) revealed mismatches of only three bases, but in comparison with *katA* gene sequence of *S. aureus* subsp. *aureus* strains (NCTC 8325 and strain Newman), 15 bases substitutions occurred within the coding region for *katA*, six of which were mis-sense mutations while the others were silent

mutations (Fig. 11, Tables 12 and 13). The substitution occurred at position no. 1099 of *katS10* gene from "C", in *katA* and *katB* genes to "T" resulted in a stop codon. The predicted protein encoded by *katS10* is 345 amino acids in length (Appendix 4).

The partial sequence of the catalase gene of other two isolates from the same disease outbreak in Alsamra village (S7, S19) in addition to 3 isolates from each of Ghanawa (G1, G11) and Alkadaro (K1, K35) slaughter houses were 100% identical to that of the corresponding region of *katS10*.

The partial sequence of the refrence strain, *S. aureus* subsp. *anaerobius* ATCC35844, DSM no. 20714, was 100% identical to that of MVF213.

The sequence results of the other nine strains: K41, S19, G2 and the reference strain are shown in Appendices 6, 7, 8 and 9, respectively.

MW G2 G35 G40 G97 k10 k11 k17 k18 k22 k41 S9 S10 S14 S16S18S19Ref[1]-ve

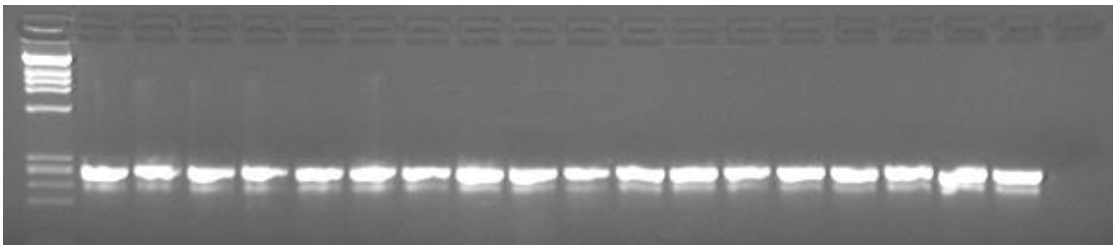


Fig. 7: a and b, agarose gel (2%) electrophoresis results of amplification of the *nuc* gene of *S. aureus* subsp. *anaerobius* isolates.

G2, G35, etc…, isolates obtained from Ghanawa Slaughter House

K1, K2, etc…, isolates obtained from Alkadaro Slaughter House

S7, S8, etc…, isolates of the outbreak of Morel's disease in Alsamra village.

Ref[1]= ATCC35844/DSM no. 20714,

Ref[2]= IBT-Göttingen Culture Collection no.9199/2628.

MW= molecular mass marker: λ DNA-Hind III/ΦXHaeIII (Finnzymes, Espoo, Finland).

MW k1 k8 k10 k11 k17 k18 k22 k41 G2 G35 G40 G58 -ve

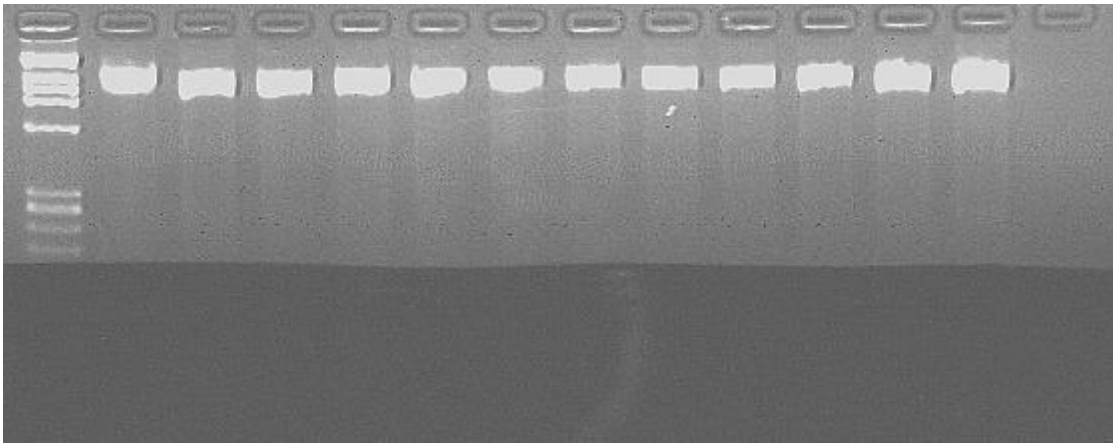


Fig. 8: Agarose gel (2%) electrophoresis results of amplification o f *kat* gene of *S. aureus* subsp. *anaerobius* isolates using primers 808F and 1583R. Abbreviations as in Fig. 7.

MW Ref^1 Ref^2 G97 S10 k1 -ve Ref^1 Ref^2 G97 S10 k1 -ve

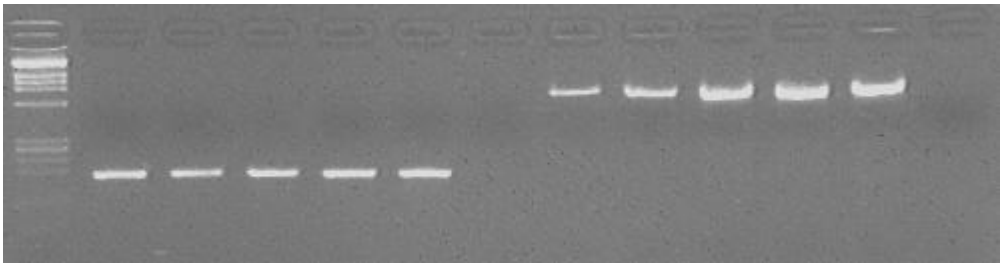
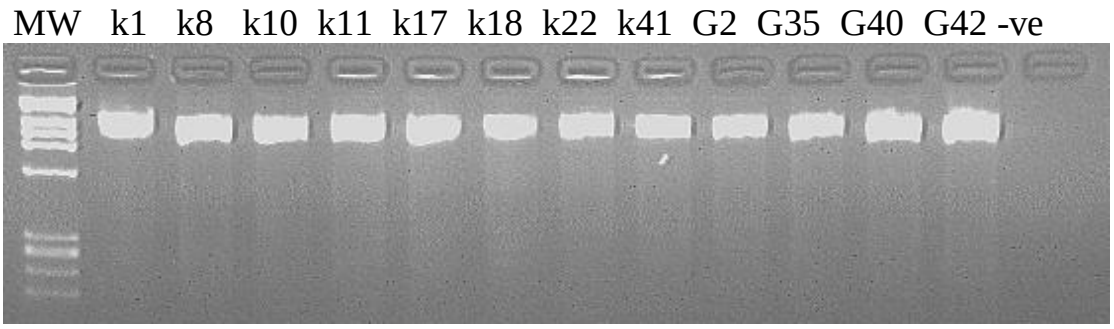


Fig. 9: Agarose gel (2%) electrophoresis results of amplification of *kat* gene of *S. aureus* subsp. *anaerobius* isolates using primers 1396F and 1583R (lanes 2, 3, 4, 5, 6, 7), 164F and 872R (lanes 8, 9, 10, 11, 12, 13). Abbreviations as in Fig. 7.

a)



b)

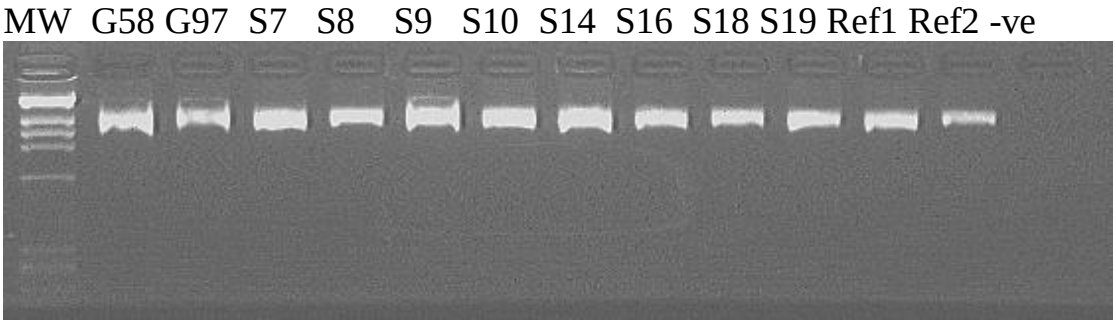


Fig. 10 a and b**:** Agarose gel (2%) electrophoresis results of amplification of *kat* gene of *S. aureus* subsp. *anaerobius* isolates using primers 164F and 1583R. Abbreviations as in Fig. 7.

Table 11: The complete sequence of the catalase-like protein gene of *S. aureus* subsp. *anaerobius* strain S10 (isolated from outbreak of Morel's disease in Alsamra village, Khartoum North Sudan)

GCTTTTTAAGTGTACTATTCAATAACTATTTAGTACTGTAAAGCGAAAAAA
ATAAAATTTTCTGATTTTTTAATCATCTTGAGCATGTTTAATTGTAATTCTG
ATGGGGTTAAATTATAATATGTATTAAATTATAATTATTATAAATTGTGGA
GGGATGACT**ATG**TCACAACAAGACAAAAAGTTAACTGGTGTTTTTGGGCA
TCCAGTATCAGATCGAGAAAATAGTATGACAGCAGGGCCTAGGGGACCTC
TTTTAATGCAAGATATTTACTTTTTAGAGCAAATGTCTCAATTTGATAGAG
AAGTAATACCAGAACGTCGAATGCATGCCAAAGGTTCTGGTGCATTTGGG
ACATTTACTGTAACTAAAGATATAACAAAATATACGAATGCTAAAATATT
CTCTGAAATAGGTAAGCAAACCGAAATGTTTGCCCGTTTCTCTACTGTAGC
AGGAGAACGTGGTGCTGCTGATGCGGAGAGTGACATTCGAGGATTTGCGT
TAAAGTTCTACACTGAAGAAGGAAACTGGGATTTAGTAGGGAATAACACA
CCAGTATTCTTCTTTAGAGATCCAAAGCTATTTGTTAGTTTAAATCGCGCG
GTGAAACGAGATCCTAGAACAAATATGAGAGATGCACAAAATAACTGGG
ATTTCTGGACGGGGCTTCCAGAAGCATTGCACCAAGTAACGATCTTAATG
TCAGATAGAGGGATTCCTAAAGATTTACGTCACATGCATGGGTTCGGTTC
ACACACATACTCTATGTATAATGATTCTGGTGAACGTGTTTGGGTTAAACT
CCATTTTAGAACGCAACAAGGTATTGAAAACTTAACTGATGAAGAAGCTG
CTGAAATTATAGCAACAGGTCGTGATTCATCTCAACGCGATTTATTCGAAG
CCATTGAAAAAGGTGATTATCCAAAATGGACAATGTATATTCAAGTAATG
ACTGAGGAACAAGCTAAAAACCATAAAGATAATCCATTTGATTTAACAAA
AGTATGGTATCACGATGAGTATCCTCTAATTGAAGTTGGAGAGTTTGAATT
AAATAGAAATCCAGATAATTACTTTATGGATGTTGAACAAGTTGCGTTTGC
ACCAACTAATATTATTCCAGGATTAGATTTTTCTCCAGACAAAATGCTGCA
AGGGCGTTTATTCTCATATGGCGATGCGCAAAGATAT**TGA**TTAGGAGTTA
ATCATTGGCAGATTCCTGTAAACCAACCTAAAGGTGTGGGTATTGAAAAT
ATTTGTCCTTTTAGTAGAGATGGTCAAATGCGCGTAGTTGACAATAACCAA
GGTGGAGGAACACATTATTATCCAAATAACCATGGTAAATTTGATTCTCA
ACCTGAATATAAAAAGCCACCATTCCCAACTGATGGATACGGCTATGAAT
ATAATCAACGTCAAGATGATGATAATTATTTTGAACAACCAGGTAAATTG
TTTAGATTACAATCAGAGGGCGCTAAAGAAAGAATTTTTACAAATACAGC
AAATGCAATGGAAGGCGTAACGGATGATGTTAAACGACGTCATATTCGTC
ATTGTTACAAAGCTGACCCAGAATATGGTAAAGGTGTTGCAAAAGCATTA
GGTATTGATATAAATTCTATTGATCTTGAAACTGAAAATGATGAAACATA
CGAAAACTTTGAAAAATAAATTTGATATGTAGTTTCTATATTGCGTAGTTG
AGCAGTTTATGA

ATG: initiation codon.

TGA: stop codon

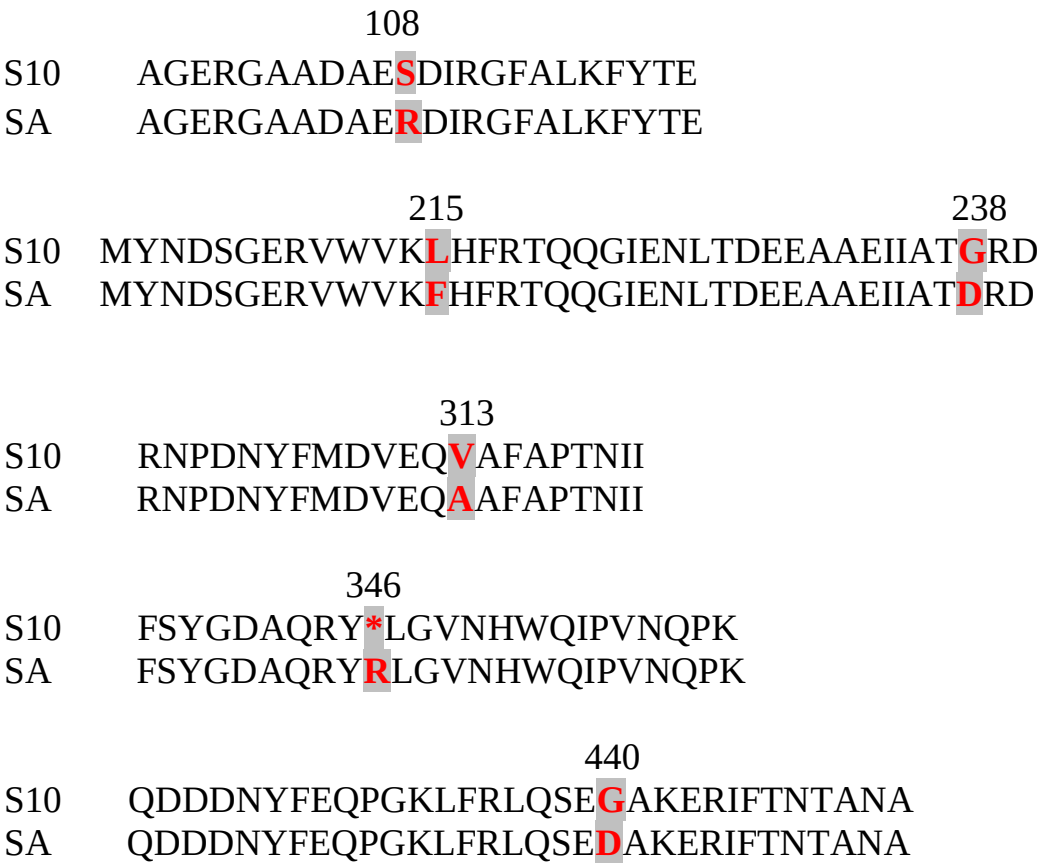


Fig. 11: Illustration of the amino acids substitutions in the catalase protein of *S. aureus* subsp. *aureus* NCTC 8325 (SA) and the deduced catalase- like protein of *S. aureus* subsp. *anaerobius* strain S10 (S10). The figures indicate the position of the amino acids.

Table 12: Nucleotide substitutions in the sequence of the catalase-like protein gene of S10 compared with that of *S. aureus* subsp. *anaerobius* MVF 213 and *S. aureus* NCTC 8325

No.	Nucletoide position	S10	MVF213	NCTC 8325
1	52	A		T
2	61	T	G	T
3	101	C		T
4	217	T		C
5	485	A		C
6	529	A		G
7	584	C		T
8	604	C		T
9	670	G		T
10	739	C		T
11	757	A		T
12	806	C		T
13	871	A		T
14	876	G		A
15	1101	T		C
16	1112	C	T	C
17	1199	T	C	C
18	1249	G		T
19	1482	G		A
20	1501	T	Deletion	T

Table 13: Amino acids resulted from nucleotide mutations in the sequence of the catalase like protein gene of S10 compared with that of *S. aureus* subsp. *anaerobius* MVF 213 and *S. aureus* NCTC 832

Nucletoide position	S10	MVF213	NCTC 8325
485	R (Arginine) (AGT)		S (Serine) (CGT)
806	L (Leucine) (CTC)		F (phenylalanine) (TTC)
876	G (Glycine) (GGT)		D (Aspartic acid)(GAT)
1101	V (Valine) (GTT)		A (Alanine) (GCT)
1112	P(Proline) (CCA)	S (Serine) (TCA)	P (Proline) (CCA)
1199	STOP CODON (TGA)	R (Arginine) (CGA)	R (Arginine) (CGA)

3.9.4 RAPD- PCR

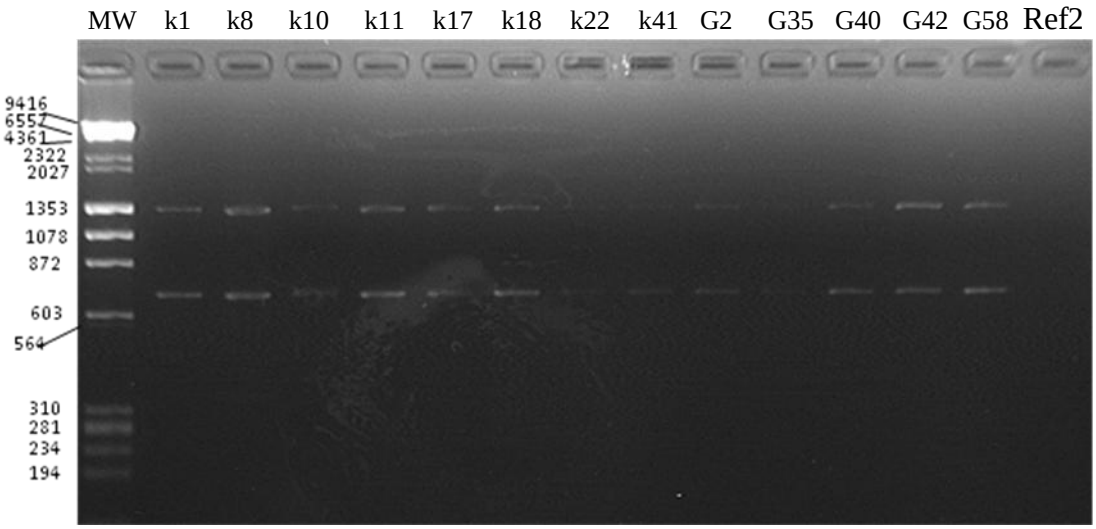
3.9.4.1 Optimization of the reaction mixture

The optimum $MgCl_2$ concentration for the RAPD-PCR test was found to be 0.75 µl per reaction and it was used for all reactions.

3.9.4.2 RAPD- PCR amplification pattern

All local isolates plus one reference strain (ATCC35844/DSM no. 20714) had identical RAPD patterns with the two primers used, but they were different from the other reference strains. Primer 786 yielded two clear bands of about 1350 and 700 bp, while primer 798 yielded 5-8 bands. A clear band was about 500. Other bands were 400, 800, 900, 1000 and 1350 bp (Fig. 12 and 13).

a)



b)

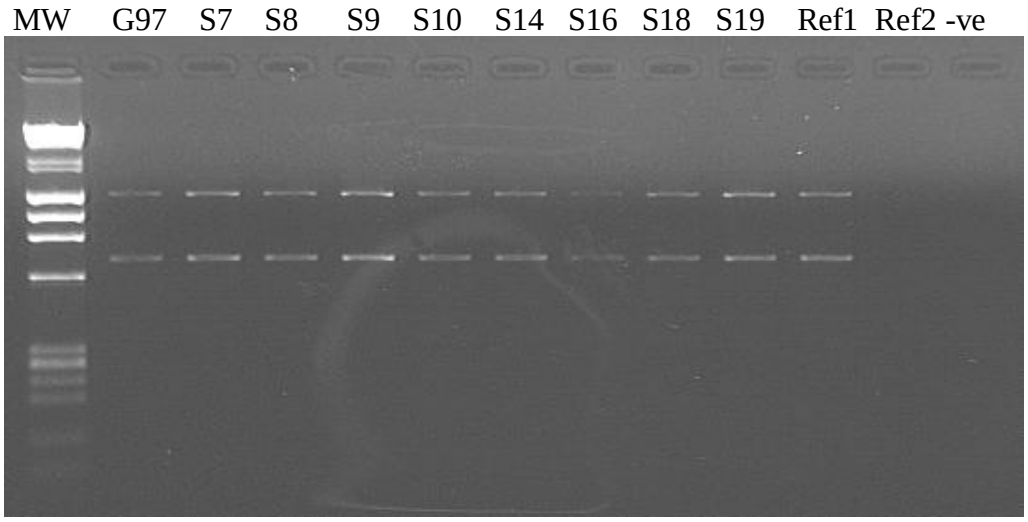
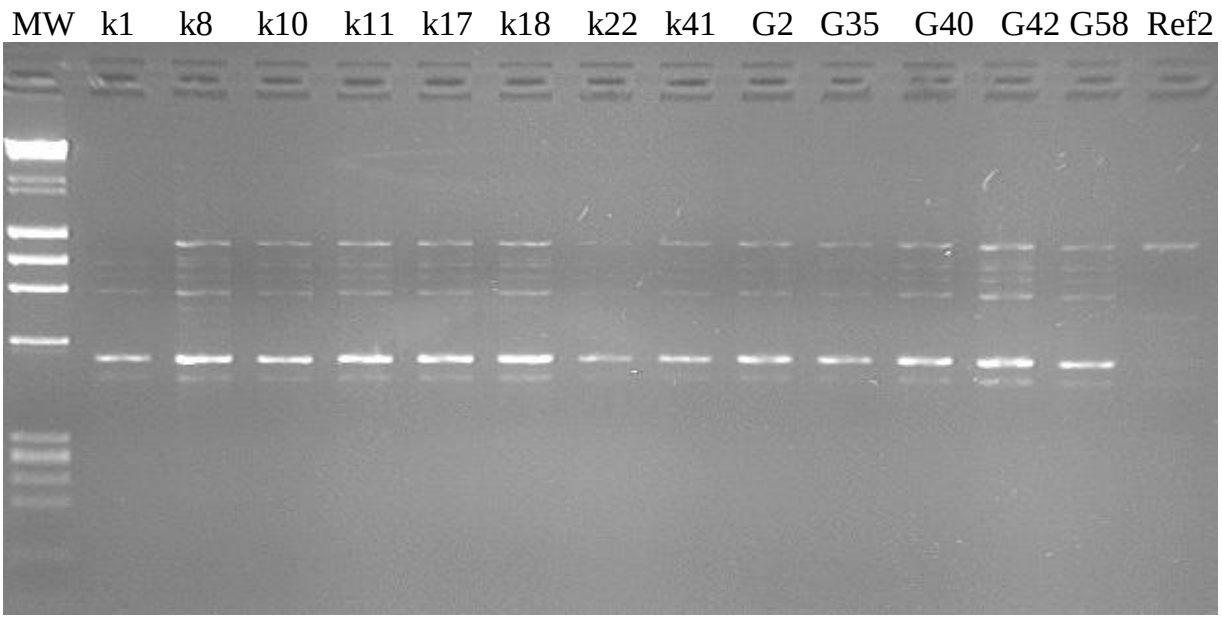


Fig. 12: Agarose gel (1%) electrophoresis results of amplification of RAPD-PCR profiles of *Staphylococcus aureus* subsp. *anaerobius* strains using primer 786. Abbreviations as in Fig. 7

a)



b)

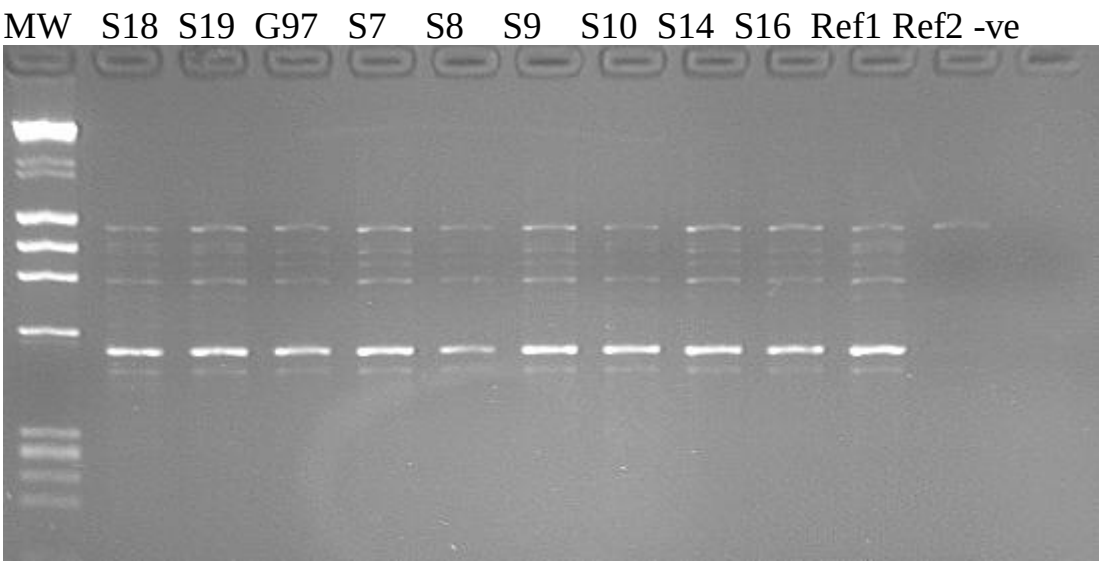


Fig. 13: Agarose gel (1%) electrophoresis results of RAPD-PCR of *Staphylococcus aureus* subsp. *anaerobius* isolates using primer 798. Abbreviations as in Fig. 7

3.9.5 Polymorphism of *coa* and *spa* gene markers

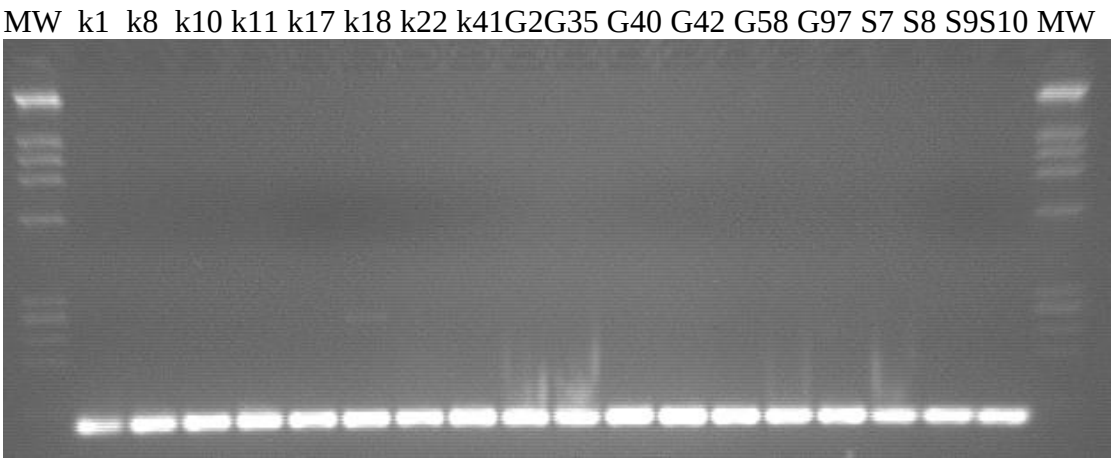
With primers for protein A encoding gene (*spa*) all local strains in addition to one reference strain (DSM no. 20714, ATCC35844) yielded amplicons of ~100 bp, while the other reference strain yielded a band of ~300 bp (Fig. 14).

With primers the coagulase gene (*coa*) all local isolates, yielded one band of about 550 bp, while one of the two reference strains yielded a band of about 600 and the other a band of about 700 bp (Fig. 15).

3.9.6 Pulsed-field gel electrophoresis (PFGE)

Pulsed-field gel electrophoresis of genomic DNA from the local strains, after digestion with restriction endonuclease *Sma*I, revealed identical restriction pattern, which was distinct for the restriction pattern of the reference strain.

a)



b)

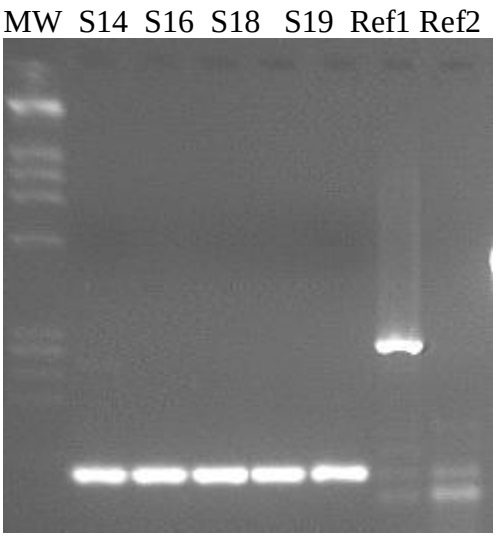
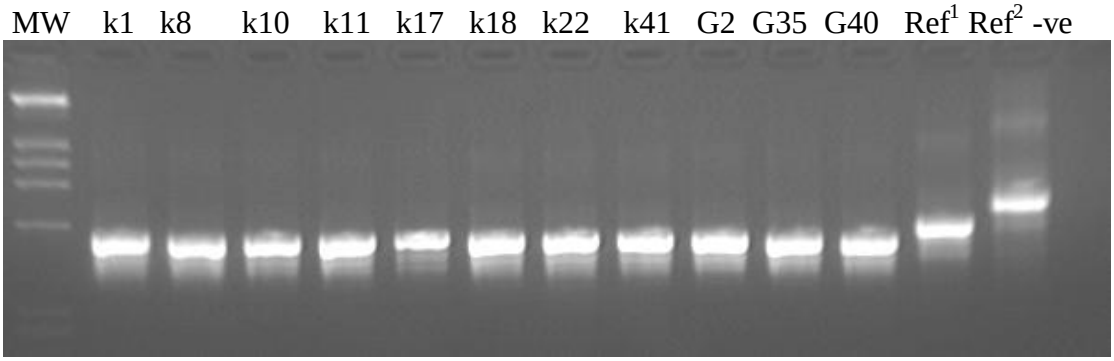


Fig. 14 a and b**:** Agarose gel (2%) electrophoresis of PCR products using primers for the *spa* gene for different *S. aureus* subsp. *anaerobius* isolates.
Abbreviations as in Fig. 7.

a)



B)

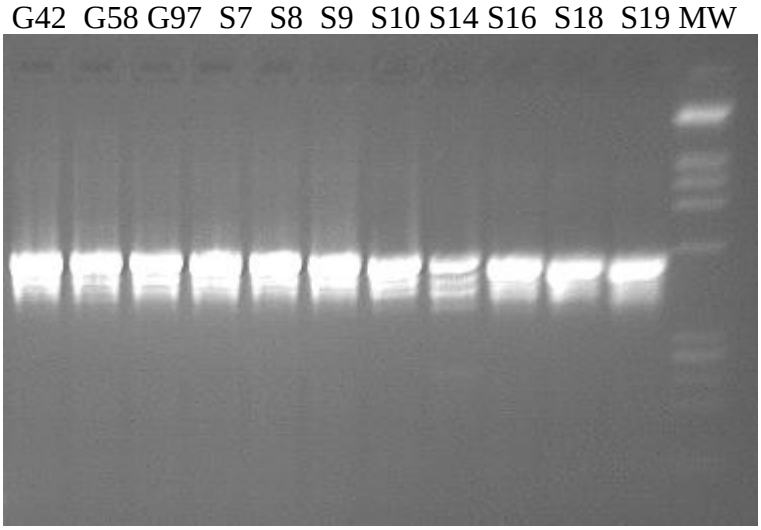


Fig. 15 a and b**:** Agarose gel (2%) electrophoresis of PCR products using primers for the *coa* gene for different *S. aureus* subsp. *anaerobius* isolates. Abbreviations as in Fig. 7.

3.4 Pathogenecity of *S. aureus* subsp. *anaerobius* and the abscess causing dose

The aim of this experiment was to confirm the ability of *S. aureus* subsp. *anaerobius* isolates to cause abscess formation in sheep before conducting the vaccination and challenge experiments. All tested inoculum sizes of *S. aureus* subsp. *anaerobius* were able to cause abscess formation. The minimum dose used was 480 cfu. Abscess formation at the site of inoculation is shown in Fig. 16.

3.5 Pathogenecity of other staphylococci

I) All animals inoculated with one species of *Staphylococcus* showed visible swellings at the sites of inoculation followed by abscess formation only in animals inoculated with *S. aureus* subsp. *anaerobius* and *S. aureus*. The size of the abscess reached up to 6.4x4.5 cm in diameter. Animals inoculated with the other species (i.e. *S. caseolyticus*, *S. lugdunensis*, *S. epidermidis* and *S. simians*) showed no abscesses formation, neither at the sites of inoculation nor in the superficial lymph nodes, but enlargement of some of these lymph nodes. In animals inoculated with *S. caseolyticus*, the right prescapular lymph node showed focal areas of caseation. Except those inoculated with *S. epidermidis* and *S. lugdunensis*, all animals showed infiltration of micro abscesses in the liver and abscesses in the lung of the animal inoculated with *S. aureus* subsp. *anaerobius* (Fig. 17 and 18, respectively). Post-mortem results of sheep inoculated with *Staphylococcus* spp. are shown in the Table 14.

II) All animals inoculated with mixture of *S. aureus anaerobius* and one of the other staphylococci showed abscesses at the inoculation sites and in the prescapular lymph nodes. While *S. aureus anaerobius* was recovered from abscesses of all animals, the other inoculated organism was recovered only from those inoculated with *S. aureus* and *S.*

caseolyticus.

Table 14: Postmortem lesions on non-vaccinated sheep after inoculation with some *Staphylococcus* spp.

	S. aureus* subsp. *anaerobius	***S. lugdunensis***	***S. caseolyticus***	***S. aureus***	***S. epidermidis***	***S. simians***
Inoculation Site	Pus, swelling	-	-	Pus, swelling	-	-
Prescapular L.N.	R/enlarged L/Haem.	R/L enlarged	R/ enlarged, caseated. L/ enlarged	-	R/ enlarged	L/ Haem.
Parotid L.N.	-	-	-	-	R/ L enlarged	R/L enlarged
Submandibular L.N.	-	-	-	-	-	L/ enlarged
Mesenteric L.N.	-	Corded	-	-	-	Enlarged and corded
Precrural	-	-	-	R/L Haem.	-	-
Popliteal L.N.	-	-	-	-	-	-
Liver	micro-abscesses, adhesion of liver and pluera	-	Micro-abscesses	Focal area of calcification	-	Micro-abscesses
Lung	Abscess	-	-	-	-	-

Fig 16: The inoculation site of sheep with different numbers of the bacterial cells (CFU) of *Staphylococcus aureus* subsp. *anaerobius*.

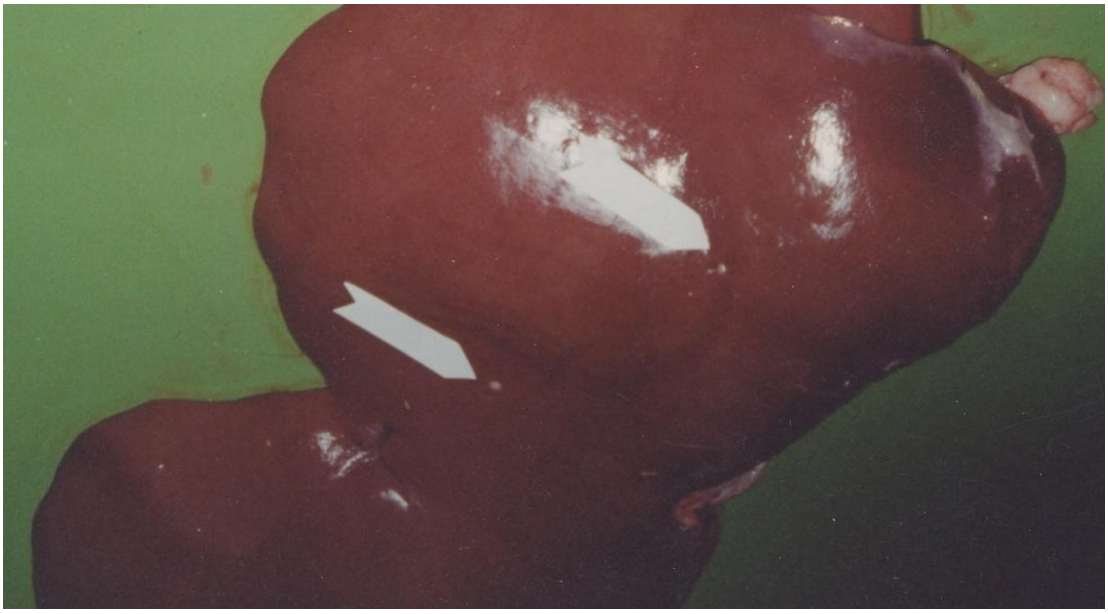


Fig.17: Micro-abscesses in the liver of ram experimentally inoculated with *Staphylococcus aureus* subsp. *anaerobius.*

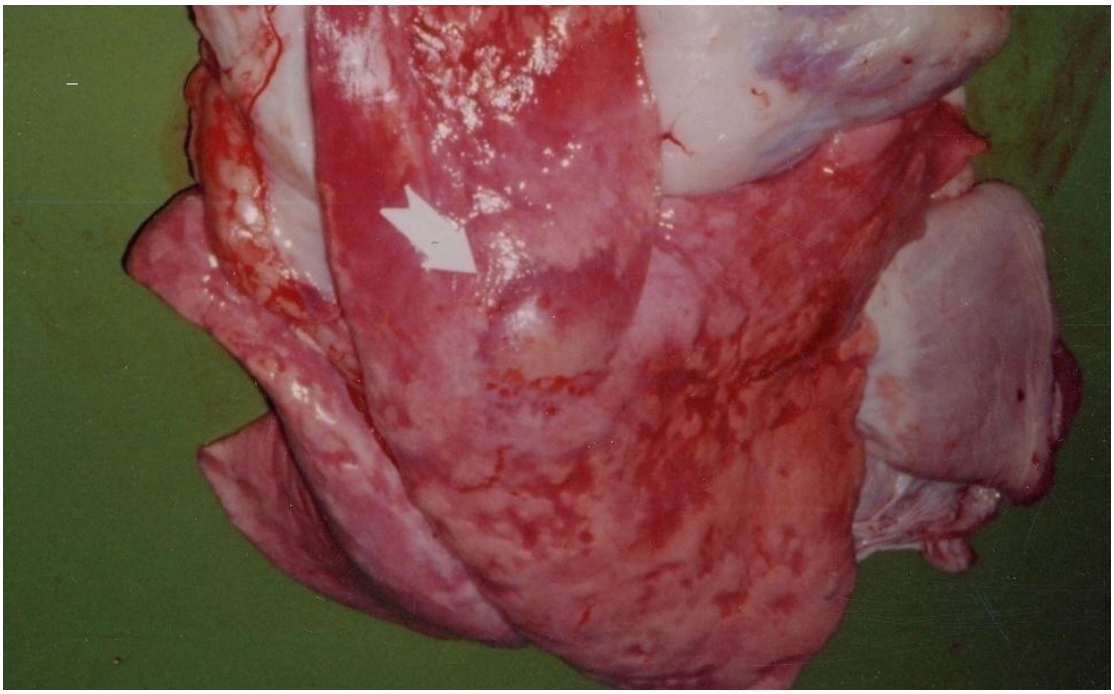


Fig. 18: Abscess formation in the lung of lamb experimentally inoculated with *Staphylococcus aureus* subsp. *anaerobius*

3.6 Determination of the effective dose of the vaccine

All vaccinated animals showed visible swellings at the sites of injection followed by increase in temperature, which dropped two days after challenge. In all vaccinated and challenged animals, except those vaccinated with 0.5 ml and boostered with 0.25 ml, some postmortem lesions could be observed in superficial lymph nodes, lung or liver (Table 15).

Table 15: Postmortem lesions of sheep inoculated with different doses of the vaccine and challenged by 1200 cfu of *Staphylococcus aureus* subsp. *anaerobius*

Animal group	Liver	Lung	Lymph nodes		
			Pre-scapular	Mes-enteric	Popliteal
A	-	Micro abscesses	Haemo-rrhagic	Enlarged	-
B	-	-	R/L Haemo-rrhagic	-	R/L Haemo-rrhagic
C	Congestion, calcification, micro-abscesses	-	-	Cording	-
D	-	-	R/ Inflammed	-	-
E	-	-	-	-	-
F	Adhesion between pleura and liver, calcification, necrotic foci	Abscessat-ion, Adhesion	Inflamed with pus	Enlarged and corded	-

A: 0.25 ml of vaccine**,** B:0.50 ml, C: 0.75 ml, D: 1.00 ml, E: 0.50 ml then 0.25 ml and F: Control

3.7 Challenge

3.7.1 Challenge using one *Staphylococcus* species

All vaccinated sheep that challenged with one species showed slight increase in temperature. The temperature increased slightly, and dropped two days after challenge. All animals showed neither abscess formation at the inoculation sites nor in the internal organs. However, animals inoculated with *S. aureus* subsp. *anaerobius* and *S. aureus* showed swelling at the sites of inoculation which decreased after 2-3 days.

Hematological parameters showed increase in neutrophils and slight decrease in PCV in all challenged groups.

3.7.2 Challenge using two *Staphylococcus* species

In animals challenged with two species of *Staphylococcus*, no abscesses formed; neither in superficial lymph nodes nor in internal organs.

Animals challenged with *S. aureus* subsp. *anaerobius* + *S. aureus* showed signs of hyper sensitivety reaction: generalized swellings and death after one day. At post-mortem, there was froth in the nostrils and mouth, subcutaneous oedema (Fig. 24, 25 and 26), congestion in the intestines (Figs. 27 and 28), brain (Fig. 29), lung, kidneys and in all lymph nodes, froth in the trachea (Fig. 30) hydroprecardium, clots in both ventricles (fibrin clot), increased synovial fluid and very flat spleen with fibrin clot.

Fig. 19: Hyper immune reaction, general swelling in lamb vaccinated with Morel's disease vaccine and challenged by *S. aureus* subsp. *anaerobius* + *S. aureus*

Fig. 20: Swelling in the chest of lamb vaccinated with Morel's disease vaccine and challenged by *S. aureus* subsp. *anaerobius* + *S. aureus*

Fig. 21: Subcutaneous oedema in lamb vaccinated with Morel's disease vaccine and challenged by *S. aureus* subsp. *anaerobius* + *S. aureus*

Fig.22: Congestion in the intestine of lamb vaccinated with Morel's disease vaccine and challenged by *S. aureus* subsp. *anaerobius* + *S. aureus*

Fig. 23: Congestion in the intestine of lamb vaccinated with Morel's disease vaccine and challenged by *S. aureus* subsp. *anaerobius* + *S. aureus*

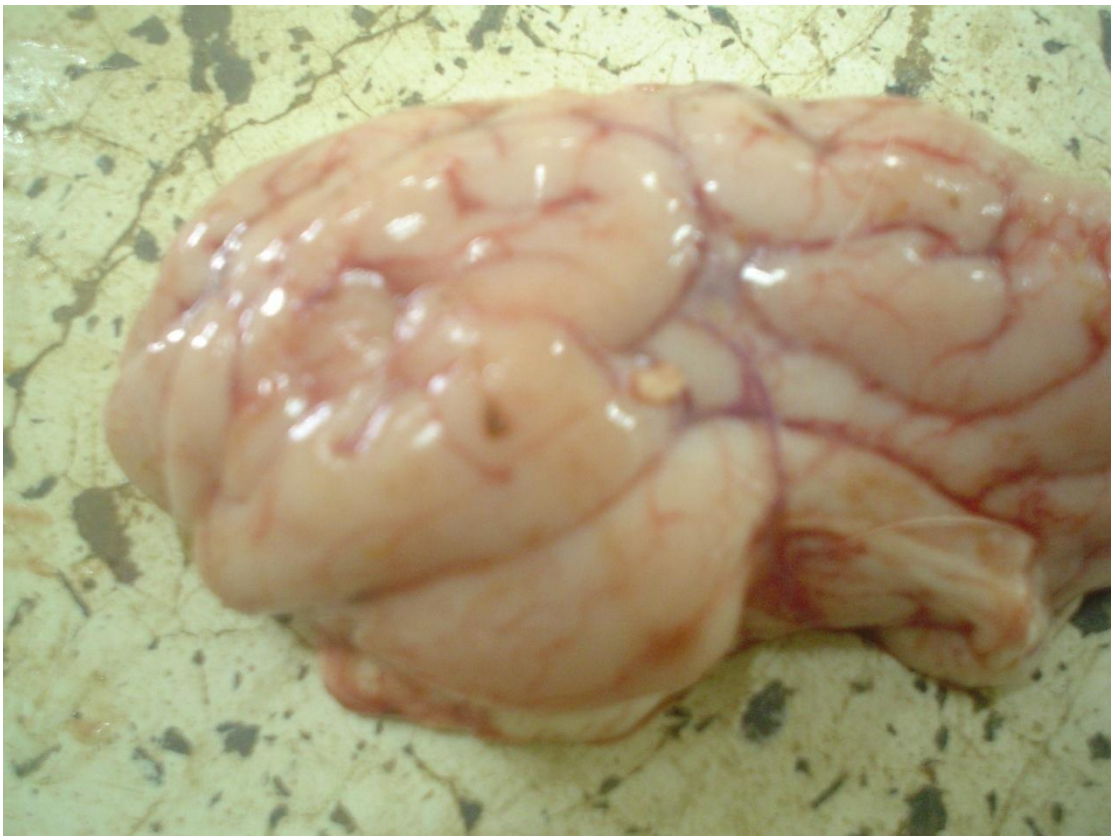


Fig. 24: Congestion in the brain of lamb vaccinated with Morel's disease vaccine and challenged by *S. aureus* subsp. *anaerobius* + *S. aureus*

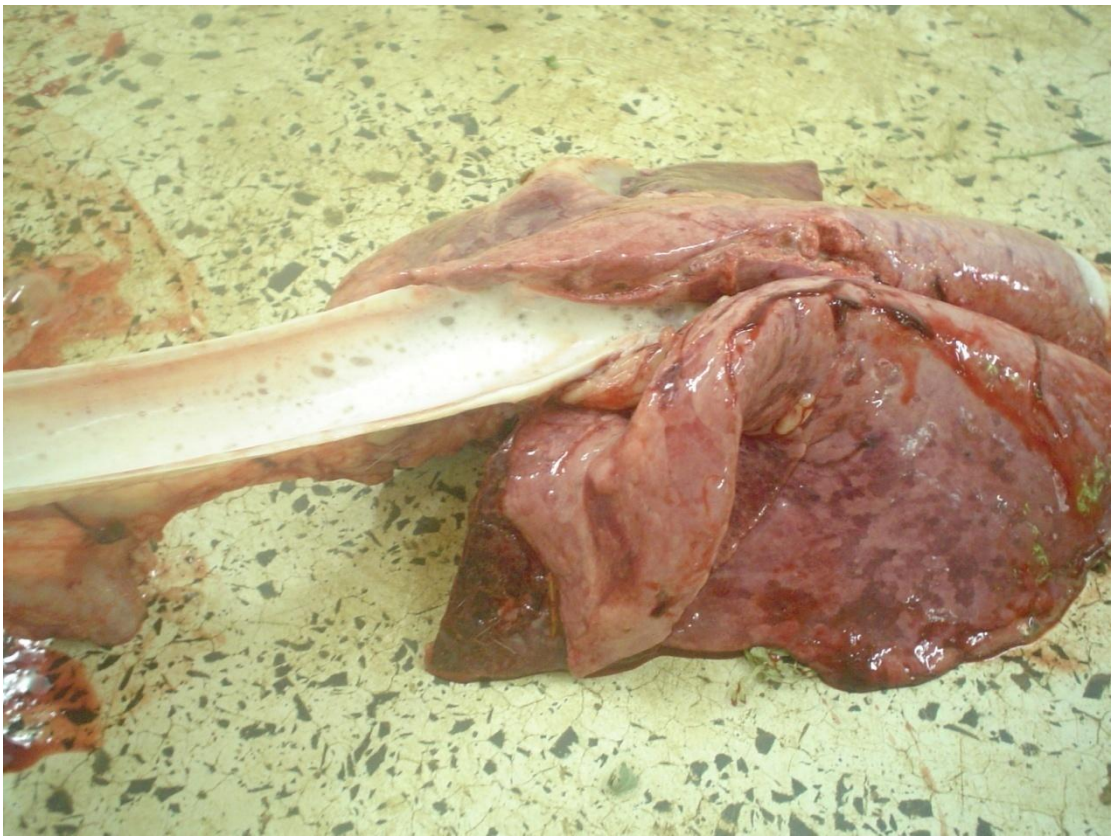


Fig. 25: Froth in the trachea of lamb vaccinated with Morel's disease vaccine and challenged by *S. aureus* subsp. *anaerobius* + *S. aureus*

3.8 Immunological tests

3.8.1 Effect of vaccination with Morel's disease vaccine on the plaque forming cell (PFCs) count

Plaques (Fig. 31) formed were counted as measure of the level of immunity conferred by immunization of sheep with Morel's disease vaccine. Significant increase ($p<0.05$) in the average number of plaques formed occurred one and two weeks after vaccination and a significantly higher ($p<0.01$) number of plaques formed one week after the booster dose (Fig. 32).

3.8.2 Effect of vaccination with Morel's disease vaccine on the splenic lymphocyte count

Splenic lymphocytes count showed slight increase ($p>0.05$) one week after vaccination, and it increased significantly ($p<0.05$) two weeks after vaccination. Booster dose of the vaccine resulted in very high increase of lymphocyte count (Fig. 3). Plaques formed were positively correlated with the lymphocytes count.

3.8.3 Validity of splenic cells

The purpose of this experiment was to determine for how long the splenic cells can remain viable before conducting the PFC assay. The splenic cells were stored normally and in two different solutions: RPMI and Histopaque at 4°C. Results are shown in Figs 35-38. While splenic cells taken from animals one week after vaccination could not remain viable more than one day, they remained viable more than 19 days when taken from animals 4 weeks after vaccination. Viability of cells was better without storage solution one week after vaccination, but it was best when stored in RPMI two weeks after vaccination. While viability was almost equal in RPMI and histopaque three weeks, it increased in Histopaque four weeks after vaccination.

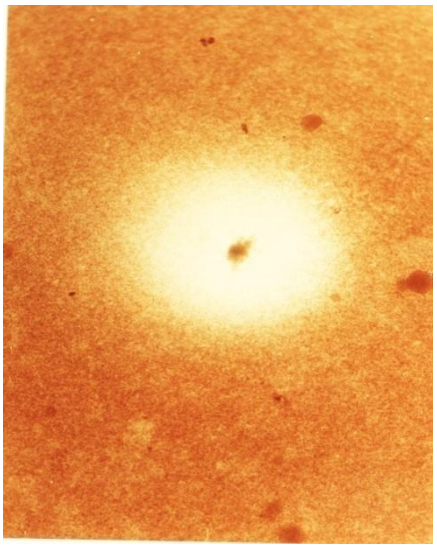
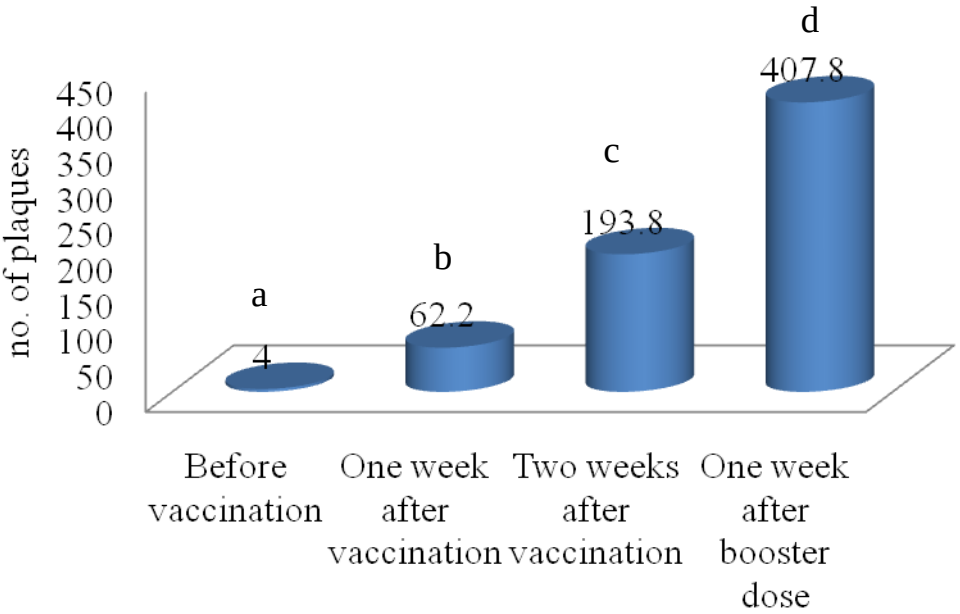


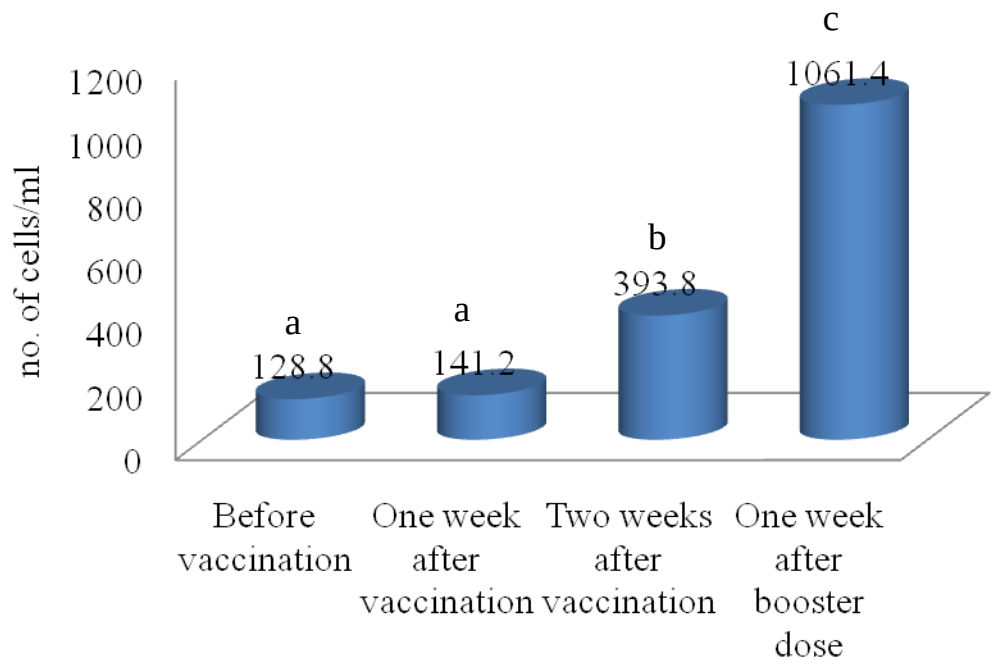
Fig. 26: Photomicrograph of typical Plaque Forming Cell. Note the single mononuclear (plasma) cell in the centre of the plaque: the erythrocytes were lysed producing holes (clear areas), 40x.

Fig. 27: Average count of plaques formed of groups of sheep vaccinated with Morel's disease vaccine



* Values with different letters are significantly different

Fig. 28: Average blood lymphocytes count of groups of sheep vaccinated with Morel's disease vaccine



* Values with different letters are significantly different

Fig. 29: Plaque forming cell assay (PFCA) and lymphocytes count, comparison between all groups

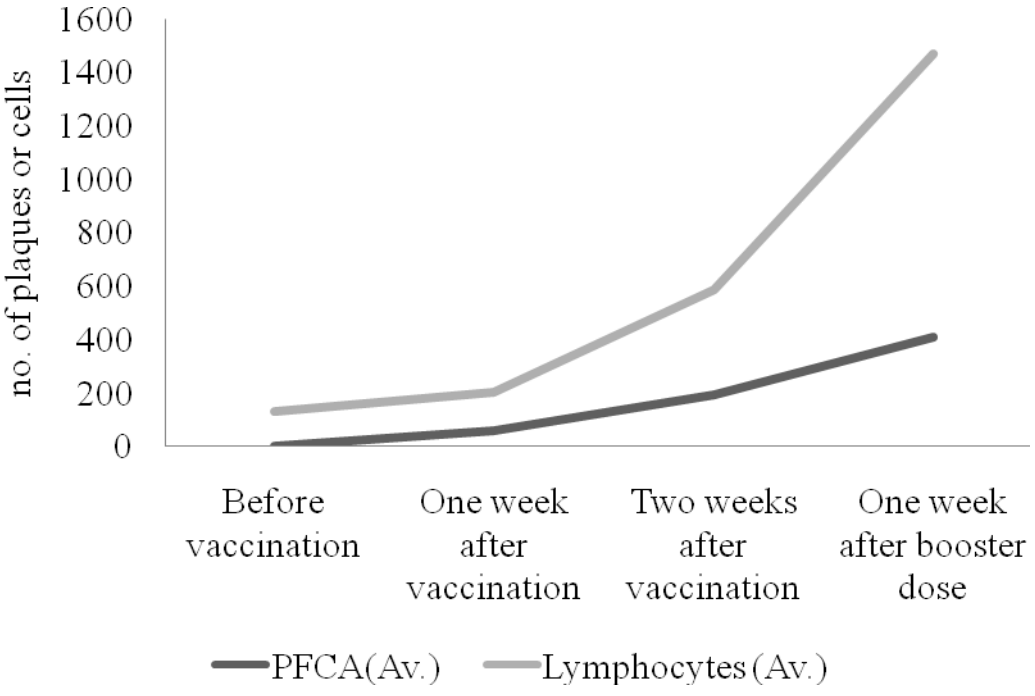


Fig. 30 Viability of splenic cells when stored normally, in RPMI or in Histopaque at 4 °C one week after vaccination

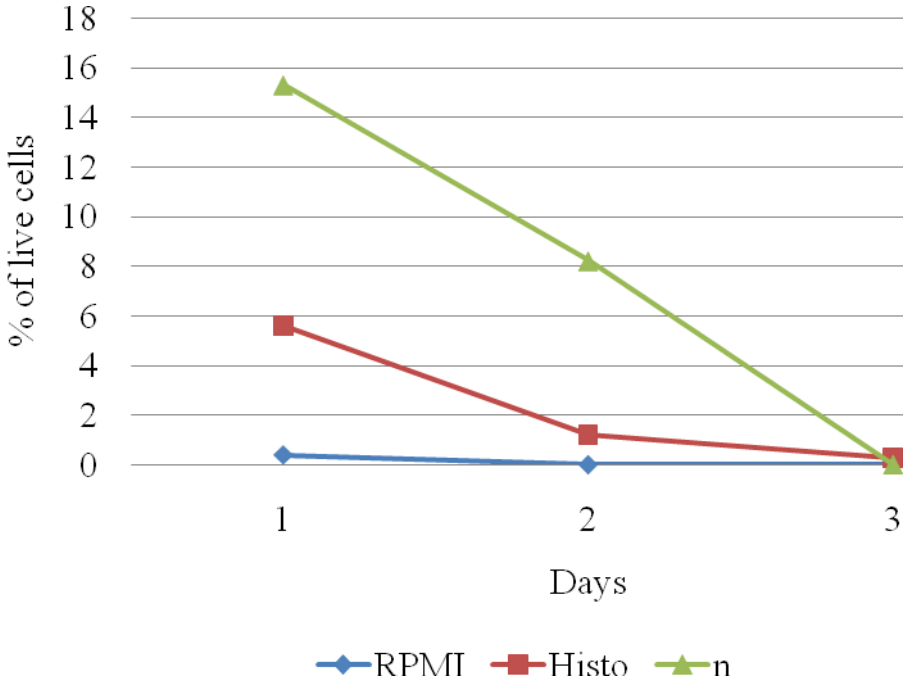


Fig. 31: Viability of splenic cells when stored normally, in RPMI or in Histopaque at 4 °C two weeks after vaccination

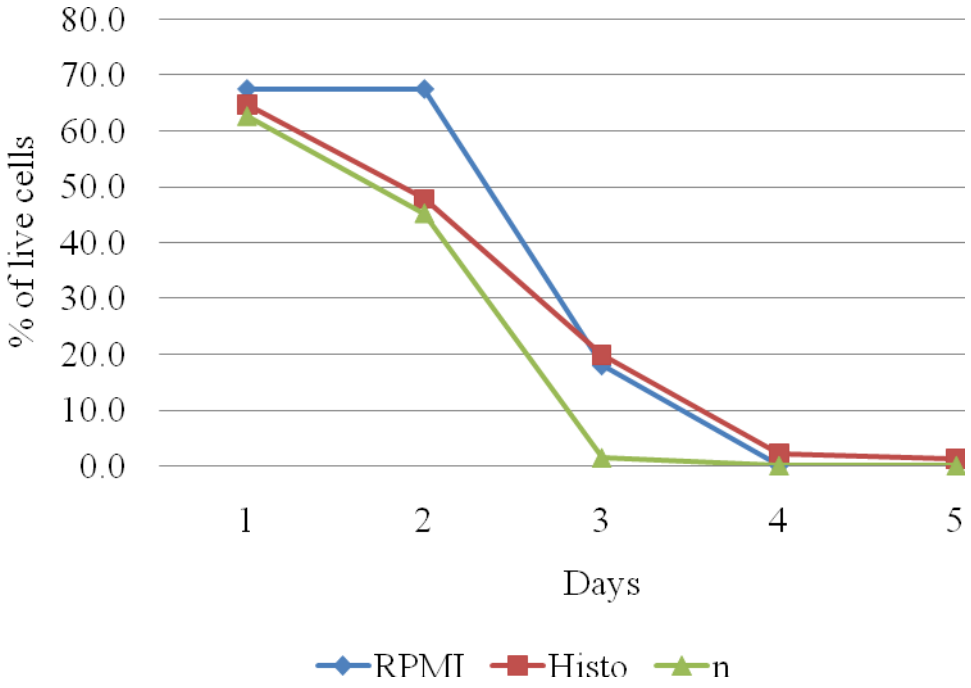


Fig. 32: Viability of splenic cells when stored normally, in RPMI or in Histopaque at 4 °C three weeks after vaccination

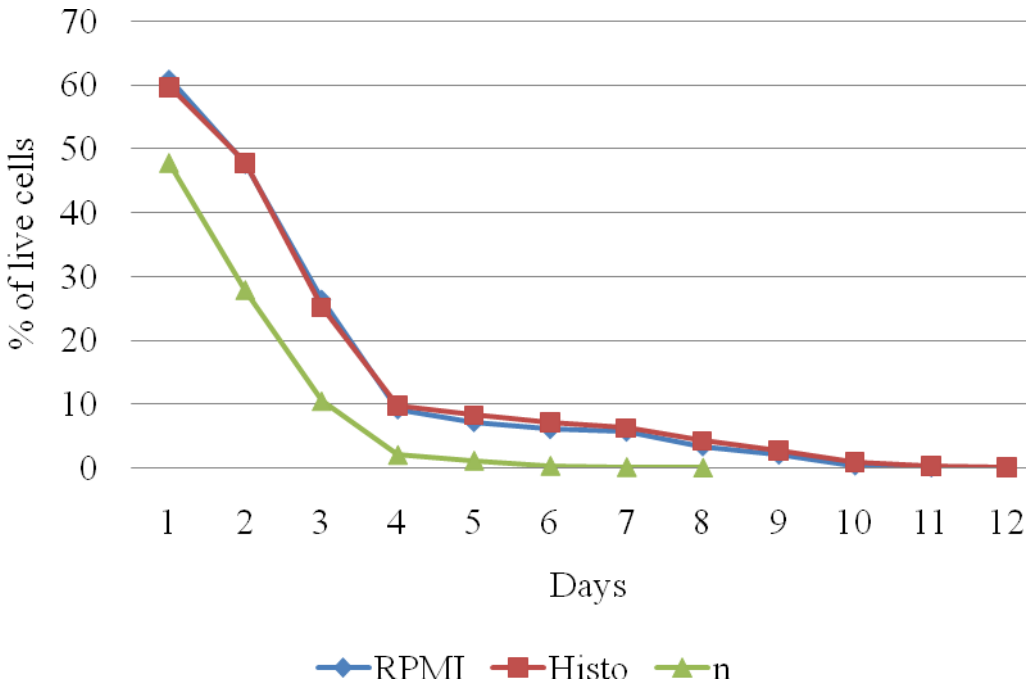
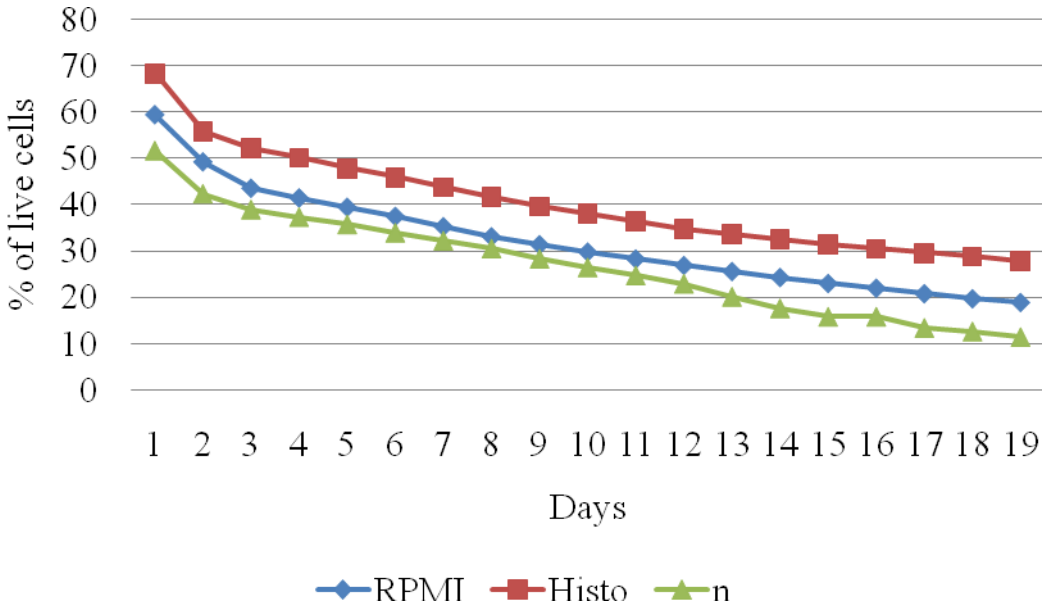


Fig. 33: Viability of splenic cells when stored normally, in RPMI or in Histopaque at 4 °C four weeks after vaccination



3.8.3. Opsonophagocytosis

This experiment was conducted to evaluate the ability of hyperimmune serum against *S. aureus* subsp. *anaerobius* to prevent abscess formation and to opsonize bacteria to help phagocytosis by PMNs. Subsutaneously inoculated bacteria two hours after opsonization produced the smallest abscess size compared with that produced by imoculation immediately after opsonization and by the control (original culture) as seen in Table 16.

Phagocytosis of opsonized bacteria by PMNs was assessed by the bacterial count. Bacterial count of that opsonized by serum taken two weeks after vaccination was lower compared with that opsonized with serum taken one week after vaccination (Tables 17 and 18). The viable count of both *S. aureus* and *S. aureus* subsp. *anaerobius* decreased after opsonization, the last count was obtained when opsonized for 2 h.

Table 16: Abscess size produced by inoculation of opsonized culture of *S. aureus* subsp. *anaerobius*

Bacterial inoculum	Abscess size (cm)
Original culture (control)	3.92x3.75
Opsonized for 0 min	2.40x2.50
Opsonized for 2 hours	1.54x1.63

Table 17: Average number of bacteria (per ml) after phagocytosis one weeks after vaccination

Opsonization time (min)	*S. aureus* subsp. *anaerobius*	*S. aureus*
Control	Uncountable	Uncountable
0	Uncountable	Uncountable
30	Uncountable	Uncountable
60	4.02×10^4	6.08×10^4
120	9.85×10^3	3.34×10^4

Table 18: Average number of bacteria (per ml) after phagocytosis two weeks after vaccination

Opsonization time (min)	*S. aureus* subsp. *anaerobius*	*S. aureus*
Control	Uncountable	Uncountable
0	Uncountable	Uncountable
30	2.35×10^3	1.1×10^3
60	1.8×10^3	8.5×10^2
120	9.5×10^2	3.5×10^2

CHAPTER FOUR

DISCUSSION

In the survey conducted in this study, *Staphylococcus* spp. were isolated in pure cultures from 68.8% of lymph nodes abscesses of sheep at meat inspection, *Corynebacterium* spp. from 26.5% and mixture of both organisms were isolated from the rest (4.7%) samples. These results suggest that staphylococci are the most prevalnt organisms that can be isolated from lymph node abscesses at meat inspection. These results are in general agreement with those obtained by Noura Karamalla (1997) and Sara Bihary (2002). Noura Karamalla (1997) isolated *Staphylococcus* spp. from 96% of the samples of lymph node abscesses of sheep at meat inspection in Alkadaro abattoir, while the rest 4% yielded mixed cultures. Sara Bihary (2002) isolated 48.7% *Staphylococcus* spp., 38.7% *Corynebacterium* spp. and 12.6% mixed culture of both *Staphylococcus* spp. and *Corynebacterium* spp. from lymph node abscesses of sheep at meat inspection in Omdurman abattoir. Among staphylococci isolated in this study, *S. aureus* subsp. *anaerobius* was the most prevalent (63.2%). This percentage of isolation is higher than those of Noura Karamalla (1997) and Sara Bihary (2002), 26% and 24%, repectively. Other staphylococci isolated were *S. caseolyticus* (21.3%), *S. aureus* (11.9%) and *S. simians*, *S. lugdunensis*, *S. warneri*, *S. epidermidis* (each 0.9%). Other than *S. aureus anaeroboius*, only two species viz: *S. caseolyticus* and *S. aureus* were among the isolates of both Noura Karamalla (1997) and Sara Bihary (2002); *S. simians* and *S. lugdunensis* were among the isolates of Sara Bihary (2002).

In outbreak of abscess disease at Alsamra village, Khartoum State, with morbidity rate of 30%, *S. aureus* subsp. *anaerobius* was isolated from 28 out of 30 (93.3%) affected sheep, while *Corynebacterium* spp.

was isolated from the rest two (6.7%) animals. These results agree with the results of previous investigators on abscess disease of sheep in the Sudan (Hamad *et al*., 1992) and earlier reports of Morel (1911), Ayuand (1923) in France in addition to a recent report made by Møller *etal*. (2000) in Denmark, who described *S. aureus* subsp. *anaerobius* as the cause of abscess disease of sheep. These results show that *Staphylococcus aureus* subsp. *anaerobius* is the most probable bacterium that can be isolated from superficial lymph node abscesses of sheep at meat inspection and the most likely cause of abscess disease of sheep in pasture.

Nuc gene encodes for the thermonuclease (TNase) enzyme produced by *Staphylococcus aureus* strains. This gene (*nuc*) has species specific sequence as was indicated by polyclonal and monoclonal antibodies to detect *S. aureus* TNase in addition to DNA hybridization test (Liebl *et al*., 1987). A primer set for the detection of the gene encoding this enzyme was designed by Brakstad *et al*. (1992), which generates a PCR product of approximately 270 bp. This primer set was used in this study in a PCR test to confirm biochemical identification of 22 representative local isolates of *S. aureus* subsp. *anaerobius*. All tested isolates were positive for this gene.

On PCR amplification of staphylococcal catalase gene (*kat*) using several primers, these local isolates also gave positive results. But, this confirmed the identification of these isolates to the species level only, not the subspecies level, i.e., they are *S. aureus*. *Staphylococcus aureus* subsp. *anaerobius* differs from *S. aureus* subsp. *aureus* in that it is catalase and benzidine negative. So, genes coding for one of these two proteins should be targeted for differentiation between the two subspecies. Catalase gene of *S. aureus* subsp. *anaerobius* (*katB*) was

found by Sanz *et al*. (2000) to have sequence differences from the prototype gene (*katA*) of *S. aureus* subsp. *aureus*. So, to confirm the identification of the local isolates, the complete catalase gene of one outbreak strain (S10) isolated in this study was sequenced.

Sequence of the putative catalase gene of *S. aureus* subsp. *anaerobius* strain S10 (SaanS10) showed 99% identity to *katB* gene of *S. aureus* subsp. *anaerobius* MVF213 (GenBank accession no. AJ000471), *katA* gene of *S. aureus* subsp. *aureus* strains NCTC 8325 and Newman (GenBank accession nos.CP000253 and AP00935.1, respectively). In comparison with *katA*, 15 bases substitutions occurred within the coding region of *katA*, six of which were mis-sense mutations while the others were silent mutations. An important substitution occurred at position no. 1099 (1036 bases upstream the initiation codon) of *katS10* gene. In *katS10* the base is "T", while in *katA* and *katB* it is "C". This substitution resulted in the code "TGA" instead of "CGA". This stop codon formula for termination of translation rendered the predicted protein to be only 345 amino acids in length. In *S. aureus* subsp. *aureus* (NCTC 8325 and Newman strains) the protein of *katA* is 505 a.a. long. Sanz *et al*. (2000) found that in *S. aureus* subsp. *anaerobius* strain MVF213, which is catalase negative, the catalase-like protein of *katB* is 445 a.a. long. Loss of the catalase activity of *S. aureus* subsp. *anaerobius* is attributed to deletion of one base 1338 nucleotides upstream the initiation codon, which resulted in shift in the reading frame and premature termination of translation 30 bases later (Sanz *et al*., 2000). In *katS10* this deletion did not occur, a feature of similarity to *katA*. The third mismatching of *katS10* and *katB* is that the substitution which occurred at base 949 upstream the initiation codon leading to serine in *katB* instead of proline in *katA* (Sanz *et al*., 2000), did not happen in *katS10*. Interestingly, all mutations

occurred in *katA* gene leading to the generation of *katB*, except the above mentioned ones, also occurred in *katS10*. This suggests that *katA* underwent mutations in at least two steps leading to the generation of *katB* and *katS10*.

To see if these mutations of *katS10* are unique features of the local strain (S10) or common to all local isolates of *S. aureus* subsp. *anaerobius*, partial sequence (about 990 bp) of the catalase gene of other eight local isolates in addition to one reference strain was performed. The segment of the gene chosen for this partial sequence targeted a region that contained most of the mutations seen in *katS10* including position 1099 of the gene. The sequence of the catalase gene of all of the Sudanese isolates was 100% identical to that of *katS10* gene, while that of the reference strain was 100% identical to *katB* sequence. These results suggest that the mutations of *katS10* may be widely present in Sudanese strains of *S. aureus* subsp. *anaerobius*, and that the strains tested could have a common origin. To confirm this assumption, further molecular characterization of the local isolates was performed. Twenty two local isolates in addition to two reference strains were analysed by RAPD-PCR and PCR amplification of two genetic markers: *spa* (staphylococcal protein A) and *coa* (colagulase) genes. With primers used for RAPD-PCR and *spa* gene all local strains in addition to one of the refrence strains was identical in the amplification pattern. But, with primers for the *coa* gene all local isolates gave similar amplification patterns, which were distinct from at least one of the reference strains. Furthermore, DNA restriction patterns in pulsed field gel electrophoresis (PFGE) of 6 local isolates and one reference strain yielded the same results. These results in conjunction with the catalase gene sequence results suggest that all Sudanese *S. aureus* subsp. *anaerobius* isolates originated from one clone. These

results are in agreement with the results obtained by El Haj and El Sanousi (2005) who reported that the local strains had similar PFGE restriction patterns and they were genetically identical.

Subcutaneous injection of non vaccinated sheep with a dose of $1.2x10^3$ cfu of *S. aureus* subsp. *anaerobius* formed abscesses reached up to 6.4x4.5 cm in size; the size measured by Hassan (1996) reached up to 6.5x6.0 cm while Sara Bihary (2002) found that the size of the formed abscesses reached 9.9x9.4 cm. This variation can be due to the differences in the inoculums sizes. However, the sizes obtained in these experimental infections was smaller from some natural cases: Møller (2000) found that it reached 15 cm in diameter, Aynaud (1927) described the size of the abscess as a size of two-hand fist, Alhendi *et al*. (1993) described it as a size of a football.

Ability to form abscesses by other staphylococci isolated in this study was also tested. Sheep inoculated with *S. aureus* formed pus discharging abscessses at the inoculation sites, haemorrhage in the left and right precrural lymph nodes. In the experiment of Sara Bihary (2002), inoculation of *S. aureus* resulted in small size subcuaneous abscess and haemorrhagic precrural lymph node. Animals inoculated with the other species (*S. caseolyticus, S. lugdunensis* and *S. simians*) showed no abscesses formation at the sites of inoculation. Sara Bihary (2002) described different sizes of abscesses formed at the sites of inoculation when she used these species for experimental infection. *S. caseolyticus* produced caseated abscess at the prescapular lymph node. Sara Bihary (2002) described abscess formation in the prescapular lymph node. Haemorrhage, enlargement of the submandibular, parotid and mesenteric lymph nodes were seen in the sheep inoculated with *S. simians*. Sara Bihary (2000) found only haemorrhagic prescapular lymph node in

animals inoculated with *S. simians*. Animals inoculated with *S. lugdunensis* showed enlargement and cording of the mesenteric lymph nodes. Sara Bihary (2002) found haemorrhagic prescapular lymph node and abscesses in the liver. Sheep inoculated with *S. epidermidis* showed enlargement of the prescapular and parotid lymph nodes. However, infiltration with micro abscesses was seen in the livers of animals inoculated with *S. caseolyticus* and *S. simians*. Failure of staphylococci (other than *S. aureus* and *S. aureus* subsp. *anaerobius*) to produce subcuataneous abscesses in sheep in this study suggests that they can not cause clinical abscess disease, but only inflammation of lymph nodes or micro abcesses in some internal organs that can be detected at meat inspection. Thes results contradict the results of Sara Bihary (2002), who showed abscesses formation in sheep inoculated with 10 different *Staphylococcus* spp. (other than *S. aureus* and *S. aureus anaerobius*) including three species isolated in this study. This may be due to the difference of inoculum sizes used in the two experiments: while the inoculum size used in this study was 1200 cfu (three times the minimum *in vitro* abscess causing dose of *S. aureus anaerobius*), Sara Bihary used inoculum size equal to Brown's opacity tube no. 4 (equivalent to about 10^9 cfu), which means one million times the minimum abscess causing dose.

Experimental inoculations of *S. aureus anaerobius* plus one of the other staphylocci isolated in this study was conducted on the assumption that there is synergistic action between *S. aureus anaerobius* and these staphylococci. This assumption was made because some of these staphylococci were isolated form abscess in mixtures with *S. aureus anaerobius*. Except in the cases of *S. aureus* and *S. caseolyticus*, none of the other staphylococci could be recovered from the abscesses developed

(at the inoculation sites and prescapular prescapular lymph nodes). Results of this experiment can augment this assumption of synergism to cause clinical abscess disease only to some extent and for only these two organisms. *S. aureus* was able to cause clinical abscess disease, and *S. caseolyticus* formed only lymph node abscess when inoculated alone.

In the present investigation, the effective dose of Morel's disease vaccine was evaluated. A dose of 0.5 ml of the vaccine boostered with 0.25 ml after fifteen days gave protection against challenge with three times the minmum abscess causing dose, as indicated by prevention of development of any lesion. Also, 1 ml of the vaccine gave protection, but prescapular lymph node was enlarged. With doses of 0.25, 0.50 and 0.75, prevention of abscess formation could occur, but inflammation of many lymph nodes had also occured. Rodwan (1996) reported that the effective dose of the vaccine was 1 ml boostered with 0.5 ml after fifteen days. These results proved the possibility of minimizing the dose to the half and giving the same protection. The positive economic impact on production of the vaccine of these results is that the same production size can be used for the double number of animals without additional costs.

When vaccinated sheep were challenged with a mixture of both *S. aureus anaerobius* and *S. aureus*, signs indicative of hyper immune reaction were seen (generalized oedema of both challenged animals and death of one animal). The protein content of Morel's disease vaccine is high (Hassan, 2000) and it is expected that it provokes both humoral and cell mediated immunity against a large number proteomes comprising its protein in vaccinated animals. Since *S. aureus anaerobius* and *S. aureus* belong to one species, it is expected that lots of antigens are shared against which immune response is elicited in animlas vaccinated with Morel's disease vaccine. The delayed type of hypersensitivity reaction

occurred in challenged lambs is thus can be attributed to the very big number of organisms used in the challenge (2,400,000 cfu), and of course the high protein concentration thereof. This reaction can not be expected to happen in nature in vaccinated animals because naturally occurring infections are caused by a very small number of organisms.

The plaque-forming cell (PCF) assay is an *in vitro* enumeration of antibody (mainly IgM) secreting cells. Hassan (2000) used this assay to compare between immune responses against *S. aureus anaerobius* in sheep vaccinated with Morel's disease vaccine and non vaccinated sheep. He found that the percetage of PFCs ranged from 75-81.59% and 0.42-2.0% for the vaccinated and non-vaccinated lambs, respectively. Hassan (2000) found this assay to be the best choice to study immunity against *S. aureus anaerobius*. So, the PFC assay was used in this study to monitor immunity against *S. aureus anaerobius* in sheep vaccinated with Morel's disease vaccine. Results of this study in PFC assay were in agreement with the findings of Hassan (2000). Furthermore, although significant increase in the number of plaques formed by spelnic cells of all groups of vaccinated sheep was seen, the number of plaques was the highest in the group given a booster dose. This result accords with the challenge experiment results, in which animals given a booster dose of the vaccine were more protected. Splenic lymphocytes also increased significantly two weeks after vaccination and the highest increase was seen one week after the booster dose. The positive correlation between the splenic lymphocyte and plaque forming cells counts indicates proliferation of cells secreting antibodies against *S. aureus anaerobius*, which conferred immunity against Morel's disease.

In practical, PFC has many limitations, one of which is the time elapse before conducting the assay after taking the spleens from animals.

To see if this assay can be conducted in another day of taking the spleens from animals, viablitiy of spleenic cells was tested when stored in two different solutions; RPMI and Histopaque at 4 °C and compared with the original buffer. Although there were differences in the ability of the solutions to keep the spelnic cells alive, and there were also differences in vibilty of splenic cells taken at different times after vaccination, but it can generally be concluded that it is better not to use storage solution for the splenic cells and to conduct the assay in the same day of taking spleens when dealing with animals one week after vaccination; to use RPMI as storage solution when dealing with animals two or three weeks after vaccination; to use Histo as storage solution when dealing with animals three or four weeks after vaccination, and to conduct the assay in the second day in when using either RPMI and Histopaqe.

Another measure of immunity against *S. aureus anaerobius* in sheep vaccinated with Morel's disease vaccine used in this study was the opsonizating ability of the hyperimmune sera to promote phagocytosis and/or killing by the complement. This was assessed by two indicatiors: viable count of bacteria after opsonization and ability of opsonized bacteria to cause abscess formation in experimental infection. The abscess size caused by inoculation of opsonized bacteria was smaller than that caused by the non-opsonized. Also, the bacterial count decreased sharply after opsonization, which positvely correlated with the time of opsoniztion. Furthermore, immune serum taken two weeks after vaccination gave better opsonization than serum taken one week after vaccination. These results are in agreement with Hassan (2000) who found significant increase in opsonizating antibodies in sera of vaccinated lambs.

However, both PFC assay and opsonophagocytosis experiments

were indicative of increased antibodies against different antigenic components of Morel's disease vaccine in sera of vaccinated animals.

While this is so, a better method for detection of immunity of vaccinated sheep against virulant staphylococci warrants future investigation.

Conclusions and recommendations

- *S. aureus* subsp. *anaerobius* is the major cause of clinical abscess disease of sheep.
- Other staphylococci can cause superficial lymph node abscesses that can be detected at meat inspection (subclinical abscess disease), but they are likley not able to cause the classical clinical subcutaneous abscess syndrome of Morel's disease.
- All Sudanese strains of *S. aureus* subsp. *anaerobius* seem to have originated from one clone and thus any one of the local stains can be used for vaccine production.
- The minimum protecting dose of Morel's disease vaccine is 0.5 ml boostered with 0.25 ml after fifteen days. Also, a single dose of 1 ml of the vaccine is effective.
- Vaccination of sheep with Morel's disease vaccine increases the concentration of antibodies raised against both *S. aureus anaerobius* and *S. aureus*.
- Although the plaque forming cell assay served as a good immunological test to monitor immunity conferred by vaccination with Morel's disease vaccine by enumerating antibody secreting cells against *S. aureus* subsp. *anaerobius*, it can not be performed effectively in live animals.

- Findig reliable immunological test(s) for the assessment of immunity level of live animals remains a challenge of future studies.

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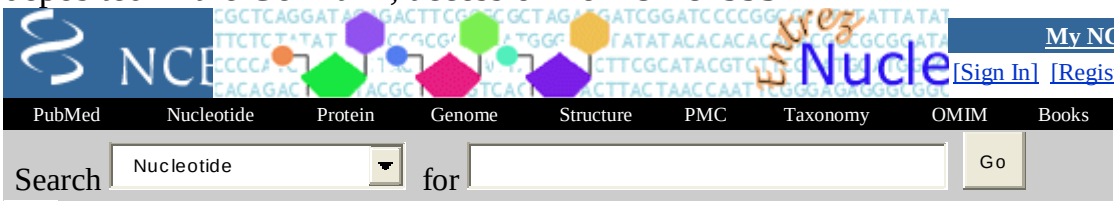
APPENDIX

Appendix 1: The complete sequence of the catalase gene of strain (S10) deposited in the GenBank, accession no EU 281993



1: EU281993. Reports Staphylococcus au...[gi:161406804] Links

- Features
- Sequence

```
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05-DEC-2007
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            protein gene, complete cds.
ACCESSION   EU281993
VERSION     EU281993.1  GI:161406804
KEYWORDS    .
SOURCE      Staphylococcus aureus subsp. anaerobius
  ORGANISM  Staphylococcus aureus subsp. anaerobius
            Bacteria; Firmicutes; Bacillales; Staphylococcus.
REFERENCE   1  (bases 1 to 1725)
  AUTHORS   Musa,N.O., Eltom,K., Babiker,A., El Sanousi,S.M.,
Gessler,F. and
            Boehnel,H.
  TITLE     Analysis of the catalase gene of a Sudanese strain of
            Staphylococcus aureus subsp. anaerobius
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1725)
  AUTHORS   Musa,N.O., Eltom,K., Babiker,A., El Sanousi,S.M.,
Gessler,F. and
            Boehnel,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (14-NOV-2007) Tropical Animal Health,
            Georg-August-University of Goettingen, Kellnerweg 6,
Goettingen
            37077, Germany
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RLFSYGDAQRY"

ORIGIN
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//
```

Appendix 2: Catalase gene of strain S10 (Sudanese strain) in comparison with the gene of the catalase-like protein of *Staphylococcus aureus subsp. anaerobius* strain MVF 213

emb|AJ000471.1|SAMVFCATA *Staphylococcus aureus* catalase gene, strain MVF213
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Score = 3162 bits (1712), Expect = 0.0
Identities = 1721/1725 (99%), Gaps = 1/1725 (0%)
Strand=Plus/Plus

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Query  61    TCTGATTTTTTAATCATCTTGAGCATGTttaattgtaattctgatggggttaaattataa  120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  63    GCTGATTTTTTAATCATCTTGAGCATGTTTAATTGTAATTCTGATGGGGTTAAATTATAA  122

Query  121   tatgtattaaattataattattataaaattGTGGAGGGATGACTATGTCACAACAAGACAA  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  123   TATGTATTAAATTATAATTATTATAAATTGTGGAGGGATGACTATGTCACAACAAGACAA  182

Query  181   AAAGTTAACTGGTGTTTTTGGGCATCCAGTATCAGATCGAGAAAATAGTATGACAGCAGG  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  183   AAAGTTAACTGGTGTTTTTGGGCATCCAGTATCAGATCGAGAAAATAGTATGACAGCAGG  242

Query  241   GCCTAGGGGACCTCTTTTAATGCAAGATATTTACTTTTTAGAGCAAATGTCTCAATTTGA  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  243   GCCTAGGGGACCTCTTTTAATGCAAGATATTTACTTTTTAGAGCAAATGTCTCAATTTGA  302

Query  301   TAGAGAAGTAATACCAGAACGTCGAATGCATGCCAAAGGTTCTGGTGCATTTGGGACATT  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  303   TAGAGAAGTAATACCAGAACGTCGAATGCATGCCAAAGGTTCTGGTGCATTTGGGACATT  362

Query  361   TACTGTAACTAAAGATATAACAAAATATACGAATGCTAAAATATTCTCTGAAATAGGTAA  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  363   TACTGTAACTAAAGATATAACAAAATATACGAATGCTAAAATATTCTCTGAAATAGGTAA  422

Query  421   GCAAACCGAAATGTTTGCCCGTTTCTCTACTGTAGCAGGAGAACGTGGTGCTGCTGATGC  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  423   GCAAACCGAAATGTTTGCCCGTTTCTCTACTGTAGCAGGAGAACGTGGTGCTGCTGATGC  482

Query  481   GGAGAGTGACATTCGAGGATTTGCGTTAAAGTTCTACACTGAAGAAGGAAACTGGGATTT  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  483   GGAGAGTGACATTCGAGGATTTGCGTTAAAGTTCTACACTGAAGAAGGAAACTGGGATTT  542

Query  541   AGTAGGGAATAACACACCAGTATTCTTCTTTAGAGATCCAAAGCTATTTGTTAGTTTAAA  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  543   AGTAGGGAATAACACACCAGTATTCTTCTTTAGAGATCCAAAGCTATTTGTTAGTTTAAA  602

Query  601   TCGCGCGGTGAAACGAGATCCTAGAACAAATATGAGAGATGCACAAAATAACTGGGATTT  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  603   TCGCGCGGTGAAACGAGATCCTAGAACAAATATGAGAGATGCACAAAATAACTGGGATTT  662

Query  661   CTGGACGGGGCTTCCAGAAGCATTGCACCAAGTAACGATCTTAATGTCAGATAGAGGGAT  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  663   CTGGACGGGGCTTCCAGAAGCATTGCACCAAGTAACGATCTTAATGTCAGATAGAGGGAT  722

Query  721   TCCTAAAGATTTACGTCACATGCATGGGTTCGGTTCACACACATACTCTATGTATAATGA  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  723   TCCTAAAGATTTACGTCACATGCATGGGTTCGGTTCACACACATACTCTATGTATAATGA  782

Query  781   TTCTGGTGAACGTGTTTGGGTTAAACTCCATTTTAGAACGCAACAAGGTATTGAAAACTT  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  783   TTCTGGTGAACGTGTTTGGGTTAAACTCCATTTTAGAACGCAACAAGGTATTGAAAACTT  842
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Query  841   AACTGATGAAGAAGCTGCTGAAATTATAGCAACAGGTCGTGATTCATCTCAACGCGATTT  900
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  843   AACTGATGAAGAAGCTGCTGAAATTATAGCAACAGGTCGTGATTCATCTCAACGCGATTT  902

Query  901   ATTCGAAGCCATTGAAAAAGGTGATTATCCAAAATGGACAATGTATATTCAAGTAATGAC  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  903   ATTCGAAGCCATTGAAAAAGGTGATTATCCAAAATGGACAATGTATATTCAAGTAATGAC  962

Query  961   TGAGGAACAAGCTAAAAACCATAAAGATAATCCATTTGATTTAACAAAAGTATGGTATCA  1020
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  963   TGAGGAACAAGCTAAAAACCATAAAGATAATCCATTTGATTTAACAAAAGTATGGTATCA  1022

Query  1021  CGATGAGTATCCTCTAATTGAAGTTGGAGAGTTTGAATTAAATAGAAATCCAGATAATTA  1080
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1023  CGATGAGTATCCTCTAATTGAAGTTGGAGAGTTTGAATTAAATAGAAATCCAGATAATTA  1082

Query  1081  CTTTATGGATGTTGAACAAGTTGCGTTTGCACCAACTAATATTATTCCAGGATTAGATTT  1140
             |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct  1083  CTTTATGGATGTTGAACAAGTTGCGTTTGCATCAACTAATATTATTCCAGGATTAGATTT  1142

Query  1141  TTCTCCAGACAAAATGCTGCAAGGGCGTTTATTCTCATATGGCGATGCGCAAAGATATTG  1200
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |
Sbjct  1143  TTCTCCAGACAAAATGCTGCAAGGGCGTTTATTCTCATATGGCGATGCGCAAAGATATCG  1202

Query  1201  ATTAGGAGTTAATCATTGGCAGATTCCTGTAAACCAACCTAAAGGTGTGGGTATTGAAAA  1260
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1203  ATTAGGAGTTAATCATTGGCAGATTCCTGTAAACCAACCTAAAGGTGTGGGTATTGAAAA  1262

Query  1261  TATTTGTCCTTTTAGTAGAGATGGTCAAATGCGCGTAGTTGACAATAACCAAGGTGGAGG  1320
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1263  TATTTGTCCTTTTAGTAGAGATGGTCAAATGCGCGTAGTTGACAATAACCAAGGTGGAGG  1322

Query  1321  AACACATTATTATCCAAATAACCATGGTAAATTTGATTCTCAACCTGAATATAAAAAGCC  1380
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1323  AACACATTATTATCCAAATAACCATGGTAAATTTGATTCTCAACCTGAATATAAAAAGCC  1382

Query  1381  ACCATTCCCAACTGATGGATACGGCTATGAATATAATCAACGTCAAGATGATGATAATTA  1440
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1383  ACCATTCCCAACTGATGGATACGGCTATGAATATAATCAACGTCAAGATGATGATAATTA  1442

Query  1441  TTTTGAACAACCAGGTAAATTGTTTAGATTACAATCAGAGGGCGCTAAAGAAAGAATTTT  1500
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1443  TTTTGAACAACCAGGTAAATTGTTTAGATTACAATCAGAGGGCGCTAAAGAAAGAATTTT  1502

Query  1501  TACAAATACAGCAAATGCAATGGAAGGCGTAACGGATGATGTTAAACGACGTCATATTCG  1560
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1503  -ACAAATACAGCAAATGCAATGGAAGGCGTAACGGATGATGTTAAACGACGTCATATTCG  1561

Query  1561  TCATTGTTACAAAGCTGACCCAGAATATGGTAAAGGTGTTGCAAAAGCATTAGGTATTGA  1620
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1562  TCATTGTTACAAAGCTGACCCAGAATATGGTAAAGGTGTTGCAAAAGCATTAGGTATTGA  1621

Query  1621  TATAAATTCTATTGATCTTGAAACTGAAAATGATGAAACATACGAAAACTTTGAAAAATA  1680
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1622  TATAAATTCTATTGATCTTGAAACTGAAAATGATGAAACATACGAAAACTTTGAAAAATA  1681

Query  1681  AATTTGATATGTAGTTTCTATATTGCGTAGTTGAGCAGTTTATGA  1725
             |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1682  AATTTGATATGTAGTTTCTATATTGCGTAGTTGAGCAGTTTATGA  1726
```

Appendix 3: Catalase gene of strain S10 in comparison with the gene of the catalase of *S.aureus* subsp. *aureus* NCTC 8325

gb|CP000253.1| *Staphylococcus aureus* subsp. *aureus* NCTC 8325, complete genome
Length=2821361
Features in this part of subject sequence: catalase
Score = 3092 bits (1674), Expect = 0.0
Identities = 1708/1725 (99%), Gaps = 0/1725 (0%)

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Query  1        GCTTTTTAAGTGTACTATTCAATAACTATTTAGTACTGTAAAGCGaaaaaaaTAAAATTT  60
               ||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
Sbjct  1269863  GCTTTTTAAGTGTACTATTCAATAACTATTTAGTACTGTAAAGCGAAAAAATTAAAATTT  1269922

Query  61       TCTGATTTTTTAATCATCTTGAGCATGTttaattgtaattctgatggggttaaattataa  120
               |||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct  1269923  TCTGATTTTTTAATCATCTTGAGCATGTTTAATTGTAATTTTGATGGGGTTAAATTATAA  1269982

Query  121      tatgtattaaattataattattataaattGTGGAGGGATGACTATGTCACAACAAGACAA  180
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1269983  TATGTATTAAATTATAATTATTATAAATTGTGGAGGGATGACTATGTCACAACAAGACAA  1270042

Query  181      AAAGTTAACTGGTGTTTTTGGGCATCCAGTATCAGATCGAGAAAATAGTATGACAGCAGG  240
               |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct  1270043  AAAGTTAACTGGTGTTTTTGGGCATCCAGTATCAGACCGAGAAAATAGTATGACAGCAGG  1270102

Query  241      GCCTAGGGGACCTCTTTTAATGCAAGATATTTACTTTTTAGAGCAAATGTCTCAATTTGA  300
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1270103  GCCTAGGGGACCTCTTTTAATGCAAGATATTTACTTTTTAGAGCAAATGTCTCAATTTGA  1270162

Query  301      TAGAGAAGTAATACCAGAACGTCGAATGCATGCCAAAGGTTCTGGTGCATTTGGGACATT  360
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1270163  TAGAGAAGTAATACCAGAACGTCGAATGCATGCCAAAGGTTCTGGTGCATTTGGGACATT  1270222

Query  361      TACTGTAACTAAAGATATAACAAAATATACGAATGCTAAAATATTCTCTGAAATAGGTAA  420
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1270223  TACTGTAACTAAAGATATAACAAAATATACGAATGCTAAAATATTCTCTGAAATAGGTAA  1270282

Query  421      GCAAACCGAAATGTTTGCCCGTTTCTCTACTGTAGCAGGAGAACGTGGTGCTGCTGATGC  480
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1270283  GCAAACCGAAATGTTTGCCCGTTTCTCTACTGTAGCAGGAGAACGTGGTGCTGCTGATGC  1270342

Query  481      GGAGAGTGACATTCGAGGATTTGCGTTAAAGTTCTACACTGAAGAAGGAAACTGGGATTT  540
               |||| ||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  1270343  GGAGCGTGACATTCGAGGATTTGCGTTAAAGTTCTACACTGAAGAAGGGAACTGGGATTT  1270402

Query  541      AGTAGGGAATAACACACCAGTATTCTTCTTTAGAGATCCAAAGCTATTTGTTAGTTTAAA  600
               |||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct  1270403  AGTAGGGAATAACACACCAGTATTCTTCTTTAGAGATCCAAAGTTATTTGTTAGTTTAAA  1270462

Query  601      TCGCGCGGTGAAACGAGATCCTAGAACAAATATGAGAGATGCACAAAATAACTGGGATTT  660
               ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1270463  TCGTGCGGTGAAACGAGATCCTAGAACAAATATGAGAGATGCACAAAATAACTGGGATTT  1270522S

Query  661      CTGGACGGGGCTTCCAGAAGCATTGCACCAAGTAACGATCTTAATGTCAGATAGAGGGAT  720
               ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1270523  CTGGACGGGTCTTCCAGAAGCATTGCACCAAGTAACGATCTTAATGTCAGATAGAGGGAT  1270582

Query  721      TCCTAAAGATTTACGTCACATGCATGGGTTCGGTTCACACACATACTCTATGTATAATGA  780
               |||||||||||||||||| |||||||||||||||| ||||||||||||||||||||||||
Sbjct  1270583  TCCTAAAGATTTACGTCATATGCATGGGTTCGGTTCTCACACATACTCTATGTATAATGA  1270642

Query  781      TTCTGGTGAACGTGTTTGGGTTAAACTCCATTTTAGAACGCAACAAGGTATTGAAAACTT  840
               |||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  1270643  TTCTGGTGAACGTGTTTGGGTTAAATTCCATTTTAGAACGCAACAAGGTATTGAAAACTT  1270702

Query  841      AACTGATGAAGAAGCTGCTGAAATTATAGCAACAGGTCGTGATTCATCTCAACGCGATTT  900
               |||||||||||||||||||||||||||||| |||| ||||||||||||||||||||||||
Sbjct  1270703  AACTGATGAAGAAGCTGCTGAAATTATAGCTACAGATCGTGATTCATCTCAACGCGATTT  1270762

Query  901      ATTCGAAGCCATTGAAAAAGGTGATTATCCAAAATGGACAATGTATATTCAAGTAATGAC  960
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1270763  ATTCGAAGCCATTGAAAAAGGTGATTATCCAAAATGGACAATGTATATTCAAGTAATGAC  1270822
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Query  961      TGAGGAACAAGCTAAAAACCATAAAGATAATCCATTTGATTTAACAAAAGTATGGTATCA  1020
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1270823  TGAGGAACAAGCTAAAAACCATAAAGATAATCCATTTGATTTAACAAAAGTATGGTATCA  1270882

Query  1021     CGATGAGTATCCTCTAATTGAAGTTGGAGAGTTTGAATTAAATAGAAATCCAGATAATTA  1080
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1270883  CGATGAGTATCCTCTAATTGAAGTTGGAGAGTTTGAATTAAATAGAAATCCAGATAATTA  1270942

Query  1081     CTTTATGGATGTTGAACAAGTTGCGTTTGCACCAACTAATATTATTCCAGGATTAGATTT  1140
                |||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  1270943  CTTTATGGATGTTGAACAAGCTGCGTTTGCACCAACTAATATTATTCCAGGATTAGATTT  1271002

Query  1141     TTCTCCAGACAAAATGCTGCAAGGGCGTTTATTCTCATATGGCGATGCGCAAAGATATTG  1200
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |
Sbjct  1271003  TTCTCCAGACAAAATGCTGCAAGGGCGTTTATTCTCATATGGCGATGCGCAAAGATATCG  1271062

Query  1201     ATTAGGAGTTAATCATTGGCAGATTCCTGTAAACCAACCTAAAGGTGTGGGTATTGAAAA  1260
                |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  1271063  ATTAGGAGTTAATCATTGGCAGATTCCTGTAAACCAACCTAAAGGTGTTGGTATTGAAAA  1271122

Query  1261     TATTTGTCCTTTTAGTAGAGATGGTCAAATGCGCGTAGTTGACAATAACCAAGGTGGAGG  1320
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1271123  TATTTGTCCTTTTAGTAGAGATGGTCAAATGCGCGTAGTTGACAATAACCAAGGTGGAGG  1271182

Query  1321     AACACATTATTATCCAAATAACCATGGTAAATTTGATTCTCAACCTGAATATAAAAAGCC  1380
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1271183  AACACATTATTATCCAAATAACCATGGTAAATTTGATTCTCAACCTGAATATAAAAAGCC  1271242

Query  1381     ACCATTCCCAACTGATGGATACGGCTATGAATATAATCAACGTCAAGATGATGATAATTA  1440
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1271243  ACCATTCCCAACTGATGGATACGGCTATGAATATAATCAACGTCAAGATGATGATAATTA  1271302

Query  1441     TTTTGAACAACCAGGTAAATTGTTTAGATTACAATCAGAGGGCGCTAAAGAAAGAATTTT  1500
                |||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct  1271303  TTTTGAACAACCAGGTAAATTGTTTAGATTACAATCAGAGGACGCTAAAGAAAGAATTTT  1271362

Query  1501     TACAAATACAGCAAATGCAATGGAAGGCGTAACGGATGATGTTAAACGACGTCATATTCG  1560
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1271363  TACAAATACAGCAAATGCAATGGAAGGCGTAACGGATGATGTTAAACGACGTCATATTCG  1271422

Query  1561     TCATTGTTACAAAGCTGACCCAGAATATGGTAAAGGTGTTGCAAAAGCATTAGGTATTGA  1620
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1271423  TCATTGTTACAAAGCTGACCCAGAATATGGTAAAGGTGTTGCAAAAGCATTAGGTATTGA  1271482

Query  1621     TATAAATTCTATTGATCTTGAAACTGAAAATGATGAAACATACGAAAACTTTGAAAAATA  1680
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1271483  TATAAATTCTATTGATCTTGAAACTGAAAATGATGAAACATACGAAAACTTTGAAAAATA  1271542

Query  1681     AATTTGATATGTAGTTTCTATATTGCGTAGTTGAGCAGTTTATGA  1725
                |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1271543  AATTTGATATGTAGTTTCTATATTGCGTAGTTGAGCAGTTTATGA  1271587
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Appendix 4: Translated sequence of the catalase gene of strain S10 in comparison with the amino acid sequence of catalase of other *S. aureus* strains

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>    ref|NP_646038.1|  Catalase [Staphylococcus aureus subsp. aureus MW2]
 ref|YP_043399.1|  catalase [Staphylococcus aureus subsp. aureus MSSA476]
 ref|YP_493929.1|  catalase [Staphylococcus aureus subsp. aureus USA300]
 ref|YP_001246765.1|  Catalase [Staphylococcus aureus subsp. aureus JH9]
 ref|YP_001316559.1|  Catalase [Staphylococcus aureus subsp. aureus JH1]
 sp|Q6G9M4|CATA_STAAS  Catalase
 sp|Q8NWV5|CATA_STAAW  Catalase
 sp|Q5HG86|CATA_STAAC  Catalase
 sp|Q99UE2|CATA_STAAM  Catalase
 sp|Q7A5T2|CATA_STAAN  Catalase
 sp|Q2FH99|CATA_STAA3  Catalase
 sp|Q2FYU7|CATA_STAA8  Catalase
 sp|Q2YXT2|CATA_STAAB  Catalase
 dbj|BAB95086.1|  Catalase [Staphylococcus aureus subsp. aureus MW2]
 emb|CAG43052.1|  catalase [Staphylococcus aureus subsp. aureus MSSA476]
 gb|ABD20999.1|  catalase [Staphylococcus aureus subsp. aureus
USA300_FPR3757]
 gb|ABQ49189.1|  Catalase [Staphylococcus aureus subsp. aureus JH9]
 gb|ABR52272.1|  Catalase [Staphylococcus aureus subsp. aureus JH1]
Length=505
 Score = 1043 bits (2697), Expect = 0.0
 Identities = 499/505 (98%), Positives = 499/505 (98%), Gaps = 0/505 (0%)
 Frame = +2

Query  164   MSQQDKKLTGVFGHPVSDRENSMTAGPRGPLLMQDIYFLEQMSQFDREVIPERRMHAKGS  343
             MSQQDKKLTGVFGHPVSDRENSMTAGPRGPLLMQDIYFLEQMSQFDREVIPERRMHAKGS
Sbjct  1     MSQQDKKLTGVFGHPVSDRENSMTAGPRGPLLMQDIYFLEQMSQFDREVIPERRMHAKGS  60

Query  344   GAFGTFTVTKDITKYTNAKIFSEIGKQTEMFARFSTVAGERGAADAESDIRGFALKFYTE  523
             GAFGTFTVTKDITKYTNAKIFSEIGKQTEMFARFSTVAGERGAADAE DIRGFALKFYTE
Sbjct  61    GAFGTFTVTKDITKYTNAKIFSEIGKQTEMFARFSTVAGERGAADAERDIRGFALKFYTE  120

Query  524   EGNWDLVGNNTPVFFFRDPKLFVSLNRAVKRDPRTNMRDAQNNWDFWTGLPEALHQVTIL  703
             EGNWDLVGNNTPVFFFRDPKLFVSLNRAVKRDPRTNMRDAQNNWDFWTGLPEALHQVTIL
Sbjct  121   EGNWDLVGNNTPVFFFRDPKLFVSLNRAVKRDPRTNMRDAQNNWDFWTGLPEALHQVTIL  180

Query  704   MSDRGIPKDLRHMHGFGSHTYSMYNDSGERVWVKLHFRTQQGIENLTDEEAAEIIATGRD  883
             MSDRGIPKDLRHMHGFGSHTYSMYNDSGERVWVK HFRTQQGIENLTDEEAAEIIAT RD
Sbjct  181   MSDRGIPKDLRHMHGFGSHTYSMYNDSGERVWVKFHFRTQQGIENLTDEEAAEIIATDRD  240

Query  884   SSQRDLFEAIEKGDYPKWTMYIQVMTEEQAKNHKDNPFDLTKVWYHDEYPLIEVGEFELN  1063
             SSQRDLFEAIEKGDYPKWTMYIQVMTEEQAKNHKDNPFDLTKVWYHDEYPLIEVGEFELN
Sbjct  241   SSQRDLFEAIEKGDYPKWTMYIQVMTEEQAKNHKDNPFDLTKVWYHDEYPLIEVGEFELN  300

Query  1064  RNPDNYFMDVEQVAFAPTNIIPGLDFSPDKMLQGRLFSYGDAQRY*LGVNHWQIPVNQPK  1243
             RNPDNYFMDVEQ AFAPTNIIPGLDFSPDKMLQGRLFSYGDAQRY LGVNHWQIPVNQPK
Sbjct  301   RNPDNYFMDVEQAAFAPTNIIPGLDFSPDKMLQGRLFSYGDAQRYRLGVNHWQIPVNQPK  360

Query  1244  GVGIENICPFSRDGQMRVVDNNQGGGTHYYPNNHGKFDSQPEYKKPPFPTDGYGYEYNQR  1423
             GVGIENICPFSRDGQMRVVDNNQGGGTHYYPNNHGKFDSQPEYKKPPFPTDGYGYEYNQR
Sbjct  361   GVGIENICPFSRDGQMRVVDNNQGGGTHYYPNNHGKFDSQPEYKKPPFPTDGYGYEYNQR  420

Query  1424  QDDDNYFEQPGKLFRLQSEGAKERIFTNTANAMEGVTDDVKRRHIRHCYKADPEYGKGVA  1603
             QDDDNYFEQPGKLFRLQSE AKERIFTNTANAMEGVTDDVKRRHIRHCYKADPEYGKGVA
Sbjct  421   QDDDNYFEQPGKLFRLQSEDAKERIFTNTANAMEGVTDDVKRRHIRHCYKADPEYGKGVA  480

Query  1604  KALGIDINSIDLETENDETYENFEK  1678
             KALGIDINSIDLETENDETYENFEK
Sbjct  481   KALGIDINSIDLETENDETYENFEK  505
```

Appendix 5: Translated sequence of the catalase gene of S10 in comparison with the amino acid sequence of the catalase-like protein of *S. aureus subps. anaerobius* strain MVF 213

>sp|Q9L4S2|CATB_STAAA Catalase-like protein
emb|CAB76840.1| Catalase [Staphylococcus aureus]
Length=455
Score = 927 bits (2396), Expect (2) = 0.0
Identities = 443/445 (99%), Positives = 443/445 (99%), Gaps = 0/445 (0%)
Frame = +2

```
Query 164 MSQQDKKLTGVFGHPVSDRENSMTAGPRGPLLMQDIYFLEQMSQFDREVIPERRMHAKGS 343
          MSQQDKKLTGVFGHPVSDRENSMTAGPRGPLLMQDIYFLEQMSQFDREVIPERRMHAKGS
Sbjct  1   MSQQDKKLTGVFGHPVSDRENSMTAGPRGPLLMQDIYFLEQMSQFDREVIPERRMHAKGS  60

Query  344 GAFGTFTVTKDITKYTNAKIFSEIGKQTEMFARFSTVAGERGAADAESDIRGFALKFYTE  523
           GAFGTFTVTKDITKYTNAKIFSEIGKQTEMFARFSTVAGERGAADAESDIRGFALKFYTE
Sbjct  61  GAFGTFTVTKDITKYTNAKIFSEIGKQTEMFARFSTVAGERGAADAESDIRGFALKFYTE  120

Query  524 EGNWDLVGNNTPVFFFRDPKLFVSLNRAVKRDPRTNMRDAQNNWDFWTGLPEALHQVTIL  703
           EGNWDLVGNNTPVFFFRDPKLFVSLNRAVKRDPRTNMRDAQNNWDFWTGLPEALHQVTIL
Sbjct  121 EGNWDLVGNNTPVFFFRDPKLFVSLNRAVKRDPRTNMRDAQNNWDFWTGLPEALHQVTIL  180

Query  704 MSDRGIPKDLRHMHGFGSHTYSMYNDSGERVWVKLHFRTQQGIENLTDEEAAEIIATGRD  883
           MSDRGIPKDLRHMHGFGSHTYSMYNDSGERVWVKLHFRTQQGIENLTDEEAAEIIATGRD
Sbjct  181 MSDRGIPKDLRHMHGFGSHTYSMYNDSGERVWVKLHFRTQQGIENLTDEEAAEIIATGRD  240

Query 884  SSQRDLFEAIEKGDYPKWTMYIQVMTEEQAKNHKDNPFDLTKVWYHDEYPLIEVGEFELN  1063
           SSQRDLFEAIEKGDYPKWTMYIQVMTEEQAKNHKDNPFDLTKVWYHDEYPLIEVGEFELN
Sbjct 241  SSQRDLFEAIEKGDYPKWTMYIQVMTEEQAKNHKDNPFDLTKVWYHDEYPLIEVGEFELN  300

Query1064  RNPDNYFMDVEQVAFAPTNIIPGLDFSPDKMLQGRLFSYGDAQRY*LGVNHWQIPVNQPK  1243
           RNPDNYFMDVEQVAFA TNIIPGLDFSPDKMLQGRLFSYGDAQRY LGVNHWQIPVNQPK
Sbjct 301  RNPDNYFMDVEQVAFASTNIIPGLDFSPDKMLQGRLFSYGDAQRYRLGVNHWQIPVNQPK  360

Query1244  GVGIENICPFSRDGQMRVVDNNQGGGTHYYPNNHGKFDSQPEYKKPPFPTDGYGYEYNQR  1423
           GVGIENICPFSRDGQMRVVDNNQGGGTHYYPNNHGKFDSQPEYKKPPFPTDGYGYEYNQR
Sbjct 361  GVGIENICPFSRDGQMRVVDNNQGGGTHYYPNNHGKFDSQPEYKKPPFPTDGYGYEYNQR  420

Query1424  QDDDNYFEQPGKLFRLQSEGAKERI  1498
           QDDDNYFEQPGKLFRLQSEGAKERI
Sbjct 421  QDDDNYFEQPGKLFRLQSEGAKERI  445
```

Appendix 6: Partial sequence of the catalase gene of *S. aureus* subsp. anaerobius strain K41 isolated from lymph node abscess of sheep at meat inspection in Alkadaro slaughter house, Khartoum North, Sudan

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TCtACTGTAGCaGGaGAACGTGgTGCTGCTGATGCGGAGAGTGACATtCGAGGATTTGCGTTAAAGTTCTACaCTGAAGAaGGAAA
CTGGGATTTAGTAGGGAATAACACACCAGTATTCTTCTTTAGAGATCCAAAGCTATTTGTTAGTTTAAATCGCGCGGTGAAACGAG
ATCCTAGAACAAATATGAGAGATGCACAAAATAACTGGGATTTCTGGACGGGGCTTCCAGAAGCATTGCACCAAGTAACGATCTTA
ATGTCAGATAGAGGGATTCCTAAAGATTTACGTCACATGCATGGGTTCGGTTCACACACATACTCTATGTATAATGATTCTGGTGA
ACGTGTTTGGGTTAAACTCCATTTTAGAACGCAACAAGGTATTGAAAACTTAACTGATGAAGAAGCTGCTGAAATTATAGCAACAG
GTCGTGATTCATCTCAACGCGATTTATTCGAAGCCATTGAAAAAGGTGATTATCCAAAATGGACAATGTATATTCAAGTAATGACT
GAGGAACAAGCTAAAAACCATAAAGATAATCCATTTGATTTAACAAAAGTATGGTATCACGATGAGTATCCTCTAATTGAAGTTGG
AGAGTTTGAATTAAATAGAAATCCAGATAATTACTTTATGGATGTTGAACAAGTTGCGTTTGCACCAACTAATATTATTCCAGGAT
TAGATTTTTCTCCAGACAAAATGCTGCAAGGGCGTTTATTCTCATATGGCGATGCGCAAAGATATTGATTAGGAGTTAATCATTGG
CAGATTCCTGTAAACCAACCTAAAGGTGTGGGTATTGAAAATATTTGTCCTTTTAGTAGAGATGGTCAAATGCGCGTAGTTGACAA
TAACCAAGGTGGAGGAACACATTATTATCCAAATAACCATGGTAAATTTGATTCTCAACCTGAATATAAAAAGCCACCATTCCCAA
CTGATGGATACGGCTATGAATATAATCAACGTCAAGATGATGATAATTATTTTGAACAACCAGGTAAATTGTTTAGATTACAATCA
GAGGGCGCTAAAGAAAGAATTTTTACAAATACAGCAAATGCAATGGAAGGCGTAACGGATGATGTTAAACGACG
```

Appendix 7: Partial sequence of the catalase gene of *S. aureus* subsp. *anaerobius* strain S19 isolated from outbreak of Morel's disease in Alsamra village, Khartoum North, Sudan

```
AACTGgGATTTAGTAGGGAATAACACACCAGTATTCTTCTTTAGAGATCCAAAGCTATTTGTTAGTTTAAATCGCGCGGTGAAACG
AGATCCTAGAACAAATATGAGAGATGCACAAAATAACTGGGATTTCTGGACGGGGCTTCCAGAAGCATTGCACCAAGTAACGATCT
TAATGTCAGATAGAGGGATTCCTAAAGATTTACGTCACATGCATGGGTTCGGTTCACACACATACTCTATGTATAATGATTCTGGT
GAACGTGTTTGGGTTAAACTCCATTTTAGAACGCAACAAGGTATTGAAAACTTAACTGATGAAGAAGCTGCTGAAATTATAGCAAC
AGGTCGTGATTCATCTCAACGCGATTTATTCGAAGCCATTGAAAAAGGTGATTATCCAAAATGGACAATGTATATTCAAGTAATGA
CTGAGGAACAAGCTAAAAACCATAAAGATAATCCATTTGATTTAACAAAAGTATGGTATCACGATGAGTATCCTCTAATTGAAGTT
GGAGAGTTTGAATTAAATAGAAATCCAGATAATTACTTTATGGATGTTGAACAAGTTGCGTTTGCACCAACTAATATTATTCCAGG
ATTAGATTTTTCTCCAGACAAAATGCTGCAAGGGCGTTTATTCTCATATGGCGATGCGCAAAGATATTGATTAGGAGTTAATCATT
GGCAGATTCCTGTAAACCAACCTAAAGGTGTGGGTATTGAAAATATTTGTCCTTTTAGTAGAGATGGTCAAATGCGCGTAGTTGAC
AATAACCAAGGTGGAGGAACACATTATTATCCAAATAACCATGGTAAATTTGATTCTCAACCTGAATATAAAAAGCCACCATTCCC
AACTGATGGATACGGCTATGAATATAATCAACGTCAAGATGATGATAATTATTTTGAACAACCAGGTAAATTGTTTAGATTACAAT
CAGAGGGCGCTAAAGAAAGAATTTTTACAAATACAGCAAATGCAATGGAAGGCGTAACGGATGATGTTAAACGACG
```

Appendix 8: Partial sequence of the catalase gene of *S. aureus* subsp. *anaerobius* strain G2 isolated from lymph node abscess of sheep at meat inspection in Ghanawa slaughter house, Omdurman, Sudan

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CACACCAGTATTCTTCTTTAGAGATCCAAAGCTATTTGTTAGTTTAAATCGCGCGGTGAAACGAGATCCTAGAACAAATATGAGAG
ATGCACAAAATAACTGGGATTTCTGGACGGGGCTTCCAGAAGCATTGCACCAAGTAACGATCTTAATGTCAGATAGAGGGATTCCT
AAAGATTTACGTCACATGCATGGGTTCGGTTCACACACATACTCTATGTATAATGATTCTGGTGAACGTGTTTGGGTTAAACTCCA
TTTTAGAACGCAACAAGGTATTGAAAACTTAACTGATGAAGAAGCTGCTGAAATTATAGCAACAGGTCGTGATTCATCTCAACGCG
ATTTATTCGAAGCCATTGAAAAAGGTGATTATCCAAAATGGACAATGTATATTCAAGTAATGACTGAGGAACAAGCTAAAAACCAT
AAAGATAATCCATTTGATTTAACAAAAGTATGGTATCACGATGAGTATCCTCTAATTGAAGTTGGAGAGTTTGAATTAAATAGAAA
TCCAGATAATTACTTTATGGATGTTGAACAAGTTGCGTTTGCACCAACTAATATTATTCCAGGATTAGATTTTTCTCCAGACAAAA
TGCTGCAAGGGCGTTTATTCTCATATGGCGATGCGCAAAGATATTGATTAGGAGTTAATCATTGGCAGATTCCTGTAAACCAACCT
AAAGGTGTGGGTATTGAAAATATTTGTCCTTTTAGTAGAGATGGTCAAATGCGCGTAGTTGACAATAACCAAGGTGGAGGAACACA
TTATTATCCAAATAACCATGGTAAATTTGATTCTCAACCTGAATATAAAAAGCCACCATTCCCAACTGATGGATACGGCTATGAAT
ATAATCAACGTCAAGATGATGATAATTATTTTGAACAACCAGGTAAATTGTTTAGATTACAATCAGAGGGCGCTAAAGAAAGAATT
TTTACAAATACAGCAAATGCAATGGAAGGCGTAACGGATGATGTTAAACGACG
```

Appendix 9: Partial sequence of the catalase gene of *S. aureus* subsp. *anaerobius* strain ATCC35844, DSM no. 20714

CTGGGAtTTAGTAGGGAATAACACACCAGTATTCTTCTTTAGAGATCCAAAGCTATTTGTTAGTTTAAATCGCGCGGTGAAACGAG
ATCCTAGAACAAATATGAGAGATGCACAAAATAACTGGGATTTCTGGACGGGGCTTCCAGAAGCATTGCACCAAGTAACGATCTTA
ATGTCAGATAGAGGGATTCCTAAAGATTTACGTCACATGCATGGGTTCGGTTCACACACATACTCTATGTATAATGATTCTGGTGA
ACGTGTTTGGGTTAAACTCCATTTTAGAACGCAACAAGGTATTGAAAACTTAACTGATGAAGAAGCTGCTGAAATTATAGCAACAG
GTCGTGATTCATCTCAACGCGATTTATTCGAAGCCATTGAAAAAGGTGATTATCCAAAATGGACAATGTATATTCAAGTAATGACT
GAGGAACAAGCTAAAAACCATAAAGATAATCCATTTGATTTAACAAAAGTATGGTATCACGATGAGTATCCTCTAATTGAAGTTGG
AGAGTTTGAATTAAATAGAAATCCAGATAATTACTTTATGGATGTTGAACAAGTTGCGTTTGCATCAACTAATATTATTCCAGGAT
TAGATTTTTCTCCAGACAAAATGCTGCAAGGGCGTTTATTCTCATATGGCGATGCGCAAAGATATCGATTAGGAGTTAATCATTGG
CAGATTCCTGTAAACCAACCTAAAGGTGTGGGTATTGAAAATATTTGTCCTTTTAGTAGAGATGGTCAAATGCGCGTAGTTGACAA
TAACCAAGGTGGAGGAACACATTATTATCCAAATAACCATGGTAAATTTGATTCTCAACCTGAATATAAAAAGCCACCATTCCCAA
CTGATGGATACGGCTATGAATATAATCAACGTCAAGATGATGATAATTATTTTGAACAACCAGGTAAATTGTTTAGATTACAATCA
GAGGGCGCTAAAGAAAGAATTTTACAAATACAGCAAATGCAATGGAAGGCGTAACGGATGATGTTAAACGAC

Poster presented at Tropentag 2007

Tropentag, October 9-11, 2007, Witzenhausen, Germany
"Utilisation of diversity in land use systems:
Sustainable and organic approaches to meet human needs"

Outbreak of Morels Disease (Sheep Abscess disease) in the Sudan

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Abstract

We report here for an outbreak of abscess disease in a flock of sheep in Al Samra village, Khartoum North, Sudan. The flock consisted of 100 animals of different ages ranging from 4 - 12 months. The animals were free grazing during the daytime and they were kept in a pen at the evenings, where they receive some type of feed supplemented with concentrates. Thirty animals were showing one or two abscess of superficial (prescapular or parotid) lymph nodes. Abscesses were round with diameter of 4 - 10 cm, soft in consistency when palpated. All abscesses were incised following aseptical proceures (shaving, rubbing with tincture of iodine and 70% alcohol) and the contents were expelled from which samples were taken in sterile containers. The contents of almost all abscesses were odourless, viscid, yellowish white to creamy in colour and were enclosed in a thick connective tissue capsule. Bacteriological examination of the contents of abscesses of 28 (93.33 %) animals revealed pure cultures of Gram-positive cocci arranged in pairs, tetrads and clusters. Biochemical tests for these bacteria were typical to those of Staphylococcus aureus subspecies anaerobius, the aetiological agent of sheep abscess disease, which was firstly described by Morel in 1911 in France. Abscesses of the remaining two animals yielded growth of *Corynebacterium* spp., the causative agent of caseous lymphadenitis of sheep. Results of this report confirm findings of previous investigations on abscess syndromes of sheep in the Sudan, in which *Staph. aureus* subsp. *anaerobius* was found to be the first organism to be incriminated in superficial lymph node abscess in sheep, especially of small ages and in sheep in steaming up operations.

Keywords: *Corynebacterium* spp., Morels Disease, sheep abscess, *Staphylococcus aureus*

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Paper submitted to *Veterinary Microbiology* on 19.11.2008

Genes and Genome

The catalase gene differentiates between some strains of *Staphylococcus aureus* subspecies *anaerobius*

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ABSTRACT

Staphylococcus aureus subspecies *anaerobius* strain S10 was isolated from outbreak of sheep abscess disease. Sequence of the catalase gene of this strain showed 99% identity to the catalase gene (*katB*) sequence of the reference strain (*S. aureus* subsp. *anaerobius* strain MVF213) with mismatching of three base pairs. An important substitution located 1036 nucleotides upstream the initiation codon from "C" in *katB* to "T" in the catalase gene of strain S10 originated a stop codon. The deduced protein (345 amino acids) is 105 amino acids shorter than that of *katB*. Partial sequence (600 – 990 bp) of the catalase gene of other eight local isolates in addition to another reference strain (DSM no. 20714) revealed the same mutations in all local (African) strains, whereas sequence of the reference (European) strain was typical to that of *katB*. Sequence of the catalase gene of *S. aureus* subsp. *anaerobius* stain S10 was deposited in the GenBank under accession no. EU281993.

Keywords: catalase gene, sheep abscess disease, *Staphylococcus aureus* subspecies *anaerobius*.

INTRODUCTION

Anaerobic *Staphylococcus aureus* bacteria are the causal agent of sheep abscess or Morel's disease (Bajmócy *et al*., 1984, Hamad *et al*., 1992). Although these bacteria are considered apathogenic to man, a report on a case of septicaemia due to one strain of these bacteria in man has recently been published (Peake *et al*., 2006). These bacteria were separated from other *S. aureus* bacteria in a subspecies (*S. aureus* subspecies *anaerobius*) because of their negative or weak growth in normal air, lack of the catalase enzyme activity in addition to some other biochemical properties (de la Fuente *et al*., 1985). Although some strains of *S. aureus* subsp. *aureus* were reported to lack this catalase enzyme activity (Tu *et al*., 1976, Friedberg *et al*., 2003, Yelmaz *et al*., 2005), they still grow well under aerobic conditions (Grüner *et al*., 2007).

Comparative studies between the catalase genes of *S. aureus* subsp. *aureus* and *S. aureus* subsp. *anaerobius* (*katA* and *katB*, respectively) showed that *katA* had undergone mutations led to deletion of one base pair in addition to 8 silent and 6 mis-sense mutations (Sanz *et al*., 2000). The deletion resulted in shift of the reading frame and premature termination of translation with subsequent generation of *katB*, which codes for a protein 55 amino acid residues shorter than *katA*. Lack of the catalase activity of *S. aureus* subsp. *anaerobius* is attributed to some of these mutations (Sanz *et al*., 2000). Loss of the catalase enzyme activity in some strains of *S. aureus* subsp. *aureus* was also attributed to mutations of the catalase gene (*katA*). While in a methicillin resistant *S. aureus* subsp. *aureus* strain deletion of five successive base pairs leading to shift in the reading frame and premature termination of translation (Grüner *et al*., 2007), substitution of a key amino acid in the protein (histidine 58 by

atyrosine) led to inactivity of this gene in methicillin sensitive *S. aureus* subsp. *aureus* strain (Piau *et al*., 2008).

We report here for some strains of *S. aureus* subsp. *anaerobius* that harbour a catalase gene that underwent mutations other than those previously reported for the European strains.

MATERIALS AND METHODS

Bacterial strains

S. aureus subsp. *anaerobius* strain S10 (SaanS10) was isolated from superficial lymph node abscess of one lamb in a flock of sheep during outbreak of Morel's disease in Alsamra village, East Nile Province of the Sudan, as has previously been reported (Musa *et al*., 2007). Other isolates and strains used in this study were as follows: two isolates from animals in the same disease outbreak, 6 isolates from superficial lymph node abscesses of sheep at meat inspection in abattoirs located in two different areas of Khartoum State, and *S. aureus* subsp. *anaerobius* DSM no. 20714 as reference strain. Identification of the isolates was based on failure of aerobic growth within 48 h, lack of catalase activity, positive coagulase activity, in addition to the fermentation ability of some sugars. All tests were done according to standards methods (Barrow and Feltham, 1993).

DNA extraction

Genomic DNA was extracted using Axy Prep Bacterial Genomic DNA Miniprep Kit of Axygen (Bioron, Ludwigshafen, Germany) with some modifications of the manufacturer's protocol. In brief, 3-5 colonies from 48 h blood agar culture were

suspended in 150 µl of the recommended buffer. Lysis of the cells was achieved by treatment with 10 µl of 1% lysostaphin (Sigma, Taufkirchen, Germany) for 1 h at 37 °C followed by addition of 2 µl of 10% Proteinase K (Bioron) at 56 °C for 2 h. The follow-up steps were carried out according to the manufacturer's protocol.

PCR

To confirm the biochemical identification of the isolates, a conserved region of the thermonuclease gene (nuc gene) of *S. aureus* was amplified by PCR using primers and conditions previously described (Brakstad *et al*., 1992).

Sequencing of the catalase gene

In order to amplify and sequence the whole catalase gene of SaanS10 and to partially sequence the catalase gene of the other isolates, primers and conditions previously described for the amplification of *katA* and *katB* (Sanz *et al*., 2000) in addition to other primers designed for this purpose were used (Table 1). Sequencing was done by Seqlab (Göttingen, Germany). For confirmation of the sequence results, both strands were sequenced, or overlapping parts of the gene were sequenced. Sequences were edited using a software program (BioEdit, Version 7.0.5.3). Alignment and comparisons were done using the Basic Local Alignment Search Tool (BLAST) of NCBI. The resulting sequence was deposited in the GenBank under accession no. EU281993.

RESULTS AND DISCUSSION

Identification of the isolates by biochemical tests could be confirmed by PCR amplification of the nuclease and catalase genes to the species level only (i.e. *S.*

aureus) but not to the subspecies level. Further genetic characterization could be made by sequencing the catalase gene. Sequence of the putative catalase gene of *S. aureus* subsp. *anaerobius* strain S10 (SaanS10) showed 99% identity to *katB* gene of *S. aureus* subsp. *anaerobius* MVF213 (GenBank accession no. AJ000471), *katA* gene of *S. aureus* subsp. *aureus* strains NCTC 8325 and Newman (GenBank accession nos.CP000253 and AP009351.1, respectively) and some other strains. The whole amplified part of the putative catalase gene of SaanS10 (*katS10*) was 1725 nucleotides in length. Comparison of this sequence with *katB* sequence revealed mismatches of only three bases. But, in comparison with *katA* 15 bases substitutions occurred within the coding region of *katA*, six of which were mis-sense mutations while the others were silent mutations. An important substitution occurred at position no. 1099 (1036 bases upstream the initiation codon) of *katS10* gene. In *katS10* the base is “T”, while in *katA* and *katB* it is “C”. This substitution resulted in the code "TGA" instead of "CGA". This code for termination of translation rendered the predicted protein to be only 345 amino acids in length. In *S. aureus* subsp. *aureus* (NCTC 8325 and Newman strains) the protein of *katA* is 505 a.a. long. In *S. aureus* subsp. *anaerobius* strain MVF213, which is catalase negative, the catalase-like protein of *katB* is 445 a.a. long. Loss of the catalase activity of *S. aureus* subsp. *anaerobius* is attributed to deletion of one base 1338 nucleotides upstream the initiation codon, which resulted in shift in the reading frame and premature termination of translation 30 bases later (Sanz *et al*., 2000). In *katS10* this deletion is absent, a feature of similarity to *katA*. The third mismatching of *katS10* and *katB* is that the substitution which occurred at base 949 upstream the initiation codon leading to serine in *katB* instead of proline in *katA* (Sanz *et al*., 2000) did happen in *katS10*. Interestingly, all mutations, except the above mentioned ones, occurred in *katA* gene leading to the generation of *katB* did also

occur in *katS10*. This suggests that *katA* underwent mutations in at least two steps leading to the generation of *katB* and *katS10*.

To see if these mutations of *katS10* are unique features of SaanS10 or common to other *S. aureus* subsp. *anaerobius* local strains, partial sequence (600- 990 bp) of the catalase genes of other eight isolates in addition to a reference strain (*S. aureus* subsp. *anaerobius* DSM no 20714) was performed. The segment of the gene chosen for this partial sequence targeted a region that contained most of the mutations seen in *katS10* including position 1099 of the gene. The sequence of the catalase gene of all local isolates was identical to that of *katS10*, while that of the reference strain was identical to *katB* sequence.

S. aureus subsp. *anaerobius* strain MVF213 was originally isolated from lamb affected with abscess disease in Spain. The mutations found in this strain leading to the generation of *katB* were also found in three other strains isolated from lambs affected with the same disease in Spain at different years (Sanz *et al*., 2000). The Spanish strains thus seem to have originated from one clone (European clone), and the local strains harbouring *katS10* seem to originate from another genetically distinct clone (African clone). This assumption can be augmented by the results of Elhaj and El Sanousi (2005) who found that local isolates of *S. aureus* subsp. *anaerobius* were identical, but distinct from the reference strain, in the DNA restriction pattern in PFGE.

In conclusion, results of this study show clear differentiation between local and reference strains of *S. aureus* subsp. *anaerobius* on the base of the catalase gene sequence. However, the potential use of the catalase gene as gene marker for typing strains of *S. aureus* subsp. *anaerobius* requires further investigations including more

international strains of both *S. aureus* subsp. *anaerobius* and catalase negative *S. aureus* subsp. *aureus*.

To the best of our knowledge, this is the second report on the catalase-like protein gene of *S. aureus* subsp. *anaerobius* and the fourth on a non-functional catalase gene of *S. aureus* in general.

AKNOWLEDGMENTS

Dr. Muna O. Elhaj, Central Veterinary Research Laboratories centre- Soba, Sudan, provided the reference strain. This work was partially funded by University of Khartoum, Sudan and the Institute of Applied Biotechnology in the Tropics (IBT) at the University of Göttingen, Germany.

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Table 1: Oligonucleotides used in this study

Primer	Sequence	Gene	Source
Nuc F	5´ GCGATTGATGGTGATACGGTT 3´	Thermo-nuclease	Brakstad *et al.* (1992)
Nuc R	5´ AGCCAAGCCTTGACGAACTAAAGC 3´	Thermo-nuclease	Brakstad *et al.* (1992)
3 F	5´ GCTTTTTAAGTGTACTATTC 3´	Catalase	This study [a]
164 F	5´ TATAAATTGTGGAGGGATGAC 3´	Catalase	Sanz *et al*. (2000)
8 F	5´ CTCCATTTTAGAACGCAACAA 3´	Catalase	Sanz *et al*. (2000)
1396 F	5´ GATGGATACGGCTATGAATA 3´	Catalase	This study [a]
872 R	5´ GCTATAATTTCAGCAGCTTC 3´	Catalase	This study [a]
1583 R	5´ TGGGTCAGCTTTGTAACA 3´	Catalase	Sanz *et al*. (2000)
1726 R	5´ TCATAAACTGCTCAACTACGC 3´	Catalase	Sanz *et al*. (2000)

[a] The primers were designed based on the sequences of the catalase genes of *Staphylococcus aureus* strain MVF213 (GenBank accession no. AJ000471) and *S. aureus* strain ATCC12600 (GenBank accession no. AJ000472)